Sex and puberty influence the innate immune system type 1 interferon response

PhD, Division of Medicine, University College London

Student:

Kate Webb

Supervisors:

Dr John Ioannou, Prof Lucy R. Wedderburn, Dr Coziana Ciurtin, Prof Gary Butler

Declaration:

I, Kate Webb confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed……Date…….

Table of Contents

Impact statement

This work is the first to demonstrate that pre-pubertal females are inherently more primed to respond to virus than males. It is the first to report that the production of interferon alpha (IFNα) after toll-like receptor 7 (TLR7) stimulation was higher in females, regardless of puberty, but also higher after puberty, regardless of sex. Through the investigation of a unique cohort of healthy and transgender young people, as well as young girls with Turner's syndrome (TUS), a unique model was built that uncouples serum sex hormones and the number of X chromosomes for the first time in humans. Through this, novel associations between X chromosome number, and serum sex hormone have been demonstrated in the innate anti-viral immune system which may contribute to the sex differences seen in viral infection, vaccination and autoimmunity.

The unique group of young people who showed such interest and willingness to be involved in this study, provide a glimpse into these relationships in a manner that was previously only possible in murine experiments. These data not only highlight the importance of sex as an under-represented variable in immunology, but also highlight the importance of pubertal development in the immune system.

This research aimed to address one of the main research questions that young people at the ARUK Centre for Adolescent Rheumatology wanted investigated: 'Why me?'. These data start to answer that question for young women with juvenile onset SLE (jSLE) which is more common in young women after puberty. These are some of the first data reporting changes in the immune system in young people who undergo gender switching and are important in the care of transgender young people.

These data reveal novel themes in the immune-pathogenesis of jSLE. TLR7, TLR9 and the endogenous retroelement LINE1 emerge as targets for future research. A novel correlation between tetherin protein expression and IFN gene expression signature is described that may be explored further as a clinical biomarker. The importance of stratifying jSLE by IFN gene expression has been highlighted here, as well as the novel method of controlling for IFN gene expression when measuring the expression of individual genes in jSLE.

Custom flow cytometry panels were designed and optimised, and a specific Nanostring Plexset technique (that has not been previously performed in the UK) was painstakingly optimised for this project. This serves as an affordable and fast platform to perform gene expression in jSLE

and will be used in future studies. The data from this project is available and serves as a valuable resource.

This project was awarded 2nd prize at the young investigator prize for basic research at the Paediatric Rheumatology European Society Congress in 2018 in Lisbon. It was presented in a talk at the same meeting, at the main meeting. The research was awarded the Eric Bywater's prize for the best rheumatology research from the Royal Colleges of Medicine rheumatology meeting in 2018. It has been presented and well received at multiple international congresses, including the European Society for Paediatric Endocrinology in 2018 in Athens, and will be presented at the European Professional Association for Transgender Health in Rome in 2019. It has been published as an original article for publication at the Frontiers of Immunology.

Publications:

1. ***Sex and Pubertal Differences in the Type 1 Interferon Pathway Associate With Both X Chromosome Number and Serum Sex Hormone Concentration.*** **Webb K**, Peckham H, Radziszewska A, Menon M, Oliveri P, Simpson F, Deakin CT, Lee S, Ciurtin C, Butler G, Wedderburn LR, Ioannou Y**. Front Immunol.** 2019 Jan 15;9:3167.

Presentations:

1. Kate Webb, Gary Butler, Coziana Ciurtin et al. Pubertal females produce an enhanced interferon-alpha anti-viral response compared to males, which is associated with X chromosome number, and not sex hormones. Oral presentation, 2018 European Society for Paediatric Endocrinology, Athens.
2. K Webb, L Wedderburn, Y Ioannou. Juvenile Systemic Lupus Erythematosus with a baseline high interferon signature associates with increased immune cell TLR7 expression and enhanced TLR7 dependent interferon alpha production. Oral presentation Paediatric Rheumatology European Society 2018, Lisbon.
3. K Webb, L Wedderburn, Y Ioannou. Sexual differences in TLR7 driven IFNα production may explain the increased prevalence of jSLE in females after puberty. Oral presentation Paediatric Rheumatology European Society YIM 2017, Athens
4. K Webb. The role of sex and puberty in interferon alpha. Invited speaker oral presentation, South African Immunology Society Congress, Cape Town 2017.
5. K Webb. Investigating the link between puberty, the immune system and systemic lupus erythematosus. Oral presentation at South African Rheumatology and arthritis association 2017

Awards:

1. Eric Bywaters Prize, Royal College of Medicine 2018
2. 2nd prize for best scientific presentation, Paediatric Rheumatology European Society 2018

Abstract

Type 1 IFNs are an antiviral cytokine family, important in jSLE which is more common in females, around puberty. It is reported here that plasmacytoid dendritic cells (pDC) from healthy females were more activated than males, and expressed more of the anti-viral surface protein, tetherin than males, suggesting that they are more primed to react to viral ligand. In healthy volunteers, females produced more type 1 IFN after TLR7 stimulation than males, regardless of puberty. There was however an increased production of type 1 IFN after TLR7 stimulation in post pubertal volunteers, regardless of sex. A unique human model including transgender volunteers and young women with TUS revealed that TLR7 induced type 1 IFN production related to X chromosome number, and serum testosterone concentration, in a manner which differed depending on the number of X chromosomes present. Female peripheral blood mononuclear cells (PBMC) expressed more of the genes coding for RNA-sensors *RIG-1* and *MDA5* regardless of puberty, and *TLR7* gene expression was increased in post pubertal females. Therefore, females were inherently more primed to respond to viral or endogenous RNA ligand than males, and produced more type 1 IFN after TLR7 stimulation, which associated with puberty in a manner that may partly explain why a type 1 IFN mediated autoimmune disease like jSLE is more common in females after puberty. Indeed, in this study, young people with jSLE had a diminished production of IFNα after TLR9 stimulation and a decreased gene expression of *TLR9*. Conversely, TLR7 induced IFNα production was unchanged and *TLR7* gene expression was increased in jSLE patients with evidence of a high background IFN. Stratifying low disease activity patients with jSLE revealed that approximately half of these patients had an upregulated IFN score, which is less than previously reported in higher disease activity and may better represent the baseline. Stratifying by IFN score revealed that the gene expression of the endogenous retroelement *LINE1* was relatively upregulated in jSLE as compared to healthy volunteers. These findings highlight the importance of TLR7 in the female predisposition toward jSLE, especially after puberty, and reveal TLR7 and LINE1 as potential targets for future investigation.

Dedication

This work is dedicated to my partner and husband, Christopher Webb. He agreed to move abroad with me to complete this work without hesitation, and worked to support me and our darling son, Nelson, who was born during this project. I dedicate this to him with love and thanks.

Acknowledgements

I would like to express my sincere gratitude to Action Medical Research who have provided funding and support for this work. It was a great honour to receive a fellowship from this prestigious and caring charity. I would also like to thank Versus Arthritis for their pioneering support of the Centre for Adolescent Rheumatology.

I would like to thank my supervisor Dr John Ioannou for his dedication, advice and expertise. I appreciate that he committed to completing his supervision even after he left his position at UCL. He has guided me with kindness and patience. My appreciation goes to Prof Lucy Wedderburn for her meticulous attention to detail and guidance. As a paediatric rheumatologist, she was a hero to me before I started this work and meeting her and working with her has been an enormous privilege. Prof Gary Butler deserves massive thanks, for his endocrinology expertise, but also his enduring kindness and support in and beyond this project.

I would like to thank Ania Radziszewska, who's careful and patient guidance and friendship helped transform me from a clinician into a scientist. I am forever in her debt. I would like to thank Hannah Peckham who expertly and compassionately worked with young people and recruited them to this study. In addition, I would like to thank Lauren Bourke, Laura Hanns, Meredyth Wilkinson, Thomas McDonnell, Hema Chaplain, Coziana Ciurtin and Corrine Fisher at the Centre for Adolescent Rheumatology for their friendship and laboratory guidance.

I would like to thank the young people and their families who were involved in this study. They inspire me every day with their resilience, commitment and altruism.

Thanks must go to Dr Claire Deakin, and Dr Sophie lee for their invaluable statistical advice. I appreciate the UMC Utrecht Luminex facility for assistance with Luminex and the Manchester LC/MS facility. Thanks must go to Dr Paola Oliveri and Dr Fraser Simpson from the UCL Genetics department and Nanostring facility for all the work they did to help me set up and perform the Nanostring analysis.

A project of this nature, living far from family and friends is impossible without their continued love and support. Thanks must go to my friends Dr. Tracey McGuire, Dr. Caroline Gooding, Dr. Caroline Hilton, Dr. Careni Spencer and Angie Vos, without whom I could not have done this. I thank my initial mentor Prof Christiaan Scott for his continuing friendship and support. My love and thanks go to my extended Schultze, Weakley and Baldwin-Perry family. I am a product of all of them, as is this work. Most especially I thank my parents Tessa and Colin Weakley, and my darling brother Dylan Weakley.

Glossary of abbreviations

APC - Antigen presenting cells

TRAF-TNF receptor–associated factor

TRIF-TIR domain–containing adaptor-inducing IFNβ

ACR-America College of Rheumatology

AGS-Aicardi Goutierres syndrome

AIM-Absent in melanoma

AIRE- Autoimmune regulator

ALR-AIM2-like receptors

ANA-Anti-nuclear antobody

ANOVA-Analysis of variance

AntiDsDNA-anti-double stranded DNA antibodies

AP3-adaptor protein 3

a-SLE-Adult-onset SLE

BAFF-B cell activating factor

BILAG-Based Composite Lupus Assessment (BICLA

BILAG-British Isles lupus assessment group index

BTK-Bruton's tyrosine kinase

CARD- caspase recruitment domain

cGAS-cyclic GMP-AMP synthase

CLR-C-type lectin receptors

CRP-C-reactive protein

DHT-Dihydrotestosterone

ELISA-enzyme linked immunosorbence assay

ERE-endogenous retroelement

ERE-Oestrogen response element

ESR-Erythrocyte sedimentation rate

FSH-Follicle stimulating hormone

GnRH-Gonadotropin releasing hormone

GWAS-Genome wide association studies

HCQ-Hydroxychloroquine

HIV-Human immune deficiency virus

HLAB27-Human lymphocyte antigen B27

IFI6-IFN alpha-inducible protein 6

IFN stimulated regulatory elements (ISRE)

IFN-Interferon

IFNAR-IFN receptor

Ikk- IκB kinase

IL-interleukin

IPEX-Immune-dysregulation, poly-endocrinopathy, enteropathy, X linked syndrome

IRAK-Interleukin 1 associated receptor family

IRF-IFN regulatory factor

ISGF£-IFN stimulated gene factor 3

ISG-IFN stimulated gene

Jak-Janus kinase

jSLE- Juvenile onset SLE

LC/MS-liquid chromatography and tandem mass spectrometry

LC3-Microtubule-associated protein 1A/1B-light chain 3

LH-Luteinising hormone

LINE-Long interspersed nuclear elements

LPS-Lipopolysaccharide

LRR- Leucine-rich repeat

LTR-Long terminal repeat

MAVS- mitochondrial antiviral signalling protein

MDA5-Melanoma differentiation-associated protein 5

MHC- Major histocompatibility complex

mTOR-Mammalian target of rapamycin

MW- Mann Whitney U test

MyD88-Myeloid differentiation primary response protein 88

NET-Neutrophil extracellular traps

NF-Kb-nuclear factor-κB

NK-Natural killer

OPN-Osteopontin

PACSIN1- Protein kinase C and casein kinase substrate in neurons protein

PAMPS-Pathogen associated molecular patterns

PBMC-Peripheral blood mononuclear cells

pDC-plasmacytoid dendritic cells

PLSCR1-Phospholipid scramblase 1

PRR- Pattern recognition receptors

R837-Imiquimod

R848-Resiquimod

RAG - Recombination activation gene

RBP-RNA binding protein

RCPCH-Royal College of Paediatrics and Child Health

RLR-RIG-I-like receptors

RNP-Ribonucleoprotein

RSV-Respiratory syncytial virus

SCL15A4-Solute Carrier Family 15 Member 4

SHBG-Steroid hormone binding globulin

SLAM-Systemic lupus activity measure

SLE- Systemic lupus erythematosus

SLEDAI-SLE disease activity index

SLICC-Systemic lupus international collaborating clinics classification

SRI-SLE response index

STA-Signal transducer and activator of transcription

TCR- T cell receptor

TIR- Toll/IL-1 receptor/resistance

TLR-Toll-like receptor

TNF-tumour necrosis factor

TRAF-TNF receptor associated factor

TRAIL-TNF relate apoptosis inducing ligand

TRAM,-TRIF-related adaptor molecule

T-reg-T-regulatory cells

TUS-Turner's syndrome

UCLH-University College London Hospital

WCC-White cell count

Xa-active X

XCI-X chromosome inactivation

Xi-inactivated X

Xist-X-inactive-specific-transcript

List of Tables

List of Figures

Chapter 1: Introduction

Sex differences exist in the prevalence and severity of many immune mediated human diseases, including infections, malignancies, and autoimmune diseases (2, 3). Systemic lupus erythematosus (SLE) is perhaps the most obvious immune disease that is highly skewed towards the female sex(4). The sex difference in juvenile onset SLE (jSLE) is further skewed after puberty, leading to the hypothesis that female sex and puberty associate with changes in the immune system which predispose females to develop jSLE. Specifically, as type 1 interferon (IFN) is a cytokine family associated with SLE, and it is known that the toll-like receptor (TLR) 7 mediated production of IFNα is higher in females, this thesis aims to explore whether TLR7 mediated IFNα production changes over puberty in a manner which may predispose females to develop jSLE. Importantly, unique human models are required to further investigate the underlying contributions of X chromosome number and serum sex hormone concentration to the sex and pubertal differences in IFN pathway.

Sex and puberty are basic biological variables. Despite this, there are very little data regarding the influence of sex and puberty on the immune system which could help to explain the sex and developmental differences seen in immune related disease phenotypes.

In this introduction, the basic and more detailed concepts and literature around sex, puberty and the immune system will be described, with a specific focus on type 1 IFN and jSLE to develop the hypothesis that puberty associates with an increased TLR7 mediated type 1 IFNα production in females, which, if true, may predispose them to the development of jSLE.

1.1.1. The immune system

The immune system is the defence mechanism of any organism against disease. It is composed of processes and structures which essentially protect an organism from pathogens. These pathogens are varied, rapidly evolve and adapt to evade the immune system in a continuous evolutionary arms race.

In humans and other vertebrates, exposure to a new pathogen or antigen (any molecule that can be bound by a major histocompatibility complex (MHC) and presented to a T cell receptor) triggers both the innate and adaptive immune system. The innate response is rapid, with recognition of non-specific pathogen associated molecular patterns (PAMPS) by pattern recognition receptors (PRR). PRR activation results in a rapid and potent early alarm response. If the pathogen is not eliminated by this early innate response, the adaptive immune system is

recruited. Antigen presenting cells (APC) migrate to lymphoid organs to present the antigen to naïve T and B cells that recognise the antigen via their specific receptors. This results in the clonal expansion and differentiation of these cells into effector cells, which, in addition to eliminating the pathogen, confer long lasting memory immunity to the organism.

Below, a brief overview will be provided of the adaptive immune system, before focussing more on the innate immune system, specifically the anti-viral, type 1 IFN mediated response.

1.1.1.1. The adaptive immune system-brief overview

The adaptive immune system is the newer system in evolutionary terms and exists only in vertebrates. The adaptive immune system can adapt to varied challenges and develop a lasting immune memory that will protect the organism against the same pathogen in its lifetime. T cells are lymphocytes that develop in the thymus. They recognise antigen via membrane bound T cell receptors (TCR). These TCR recognise short peptide fragments of antigen, presented to them via MHC on an APC. The TCR is restricted to recognise both a particular peptide sequence and the particular MHC upon which is presented. Antigen presented on MHCI complex is recognised by the CD8 co-receptor on cytotoxic T cells, whereas antigen on MHCII is recognised by the CD4 co-receptor on helper T cells. Cytotoxic T cells largely recognise viral peptide presented via MHCI and result in the killing of the infected presenting cell. All nucleated cells express MHCI and therefore can be effectively killed. Helper CD4 T cells are capable of killing the presenting cell, but also capable of further modulating the immune response via the activation of B cells, dendritic cells and macrophages(5).

B cells produce immunoglobulins. Immunoglobulins that are bound to the B cell membrane are effectively B cell receptors (much like TCR), and the soluble immunoglobulins released by B cells are known as antibodies. Antibodies bind antigen in the extra cellular spaces of the body and mark the antigen (and associated pathogen) for destruction by other cells. Antibodies may also undergo secondary diversification via somatic hyper-mutation, gene conversion and class switching.

Each human has an immensely diverse repertoire of TCR and antibody due to somatic VD(J) recombination under the guidance of recombination activation gene enzymes (RAG). In the thymus, the autoimmune regulator (AIRE) protein is responsible for the specific deletion of self-reactive T cells(5).

In the adaptive immune system, there is an initial maturation of response after the first exposure to a pathogen, and a rapid, specific and effective response upon subsequent exposures, swiftly eliminating the pathogen before it can establish infection.

Many of the discoveries of the immune system come from the study of diseases with abnormalities in the immune response. Charles Janeway Jr., the author of the famous Janeway's Immunobiology textbook, in one of his final papers, reminisced about how his father, an Immunologist, described the emergence of a clinical syndrome in infants lacking immunoglobulin, after the advent of penicillin, where previously they would not have survived(6, 7). The discovery of this immune abnormality, along with others, has shaped of our knowledge about how the immune system works, and this theme will be echoed throughout the introduction. In the same paper, Janeway commented that the adaptive immune system was solely responsible for diseases manifesting with immune deficiency and autoimmunity(6). In recent years, as more of the innate immune system is discovered, it is emerging that the distinction between the two is probably more blurred than was previously thought. With the emergence of transcriptome and genome sequencing, as discussed later, it is becoming clear that many of the diseases that were thought to be largely due to dysfunction of the adaptive immune system, like SLE, may have a more innate basis(1).

1.1.1.2. The innate immune system-brief overview

The innate immune system is the more recently discovered, although more evolutionarily ancient, arm of immune defence. It is based on the principle that there are conserved non-self-molecules that trigger a non-specific alarm via germ line encoded PRR. This principle of 'pattern recognition' was proposed by the same Charles Janeway Jr. at a symposium in 1989, and describes two features of innate immunity: the ability to distinguish between self and pathogen non-self and the ability to activate the adaptive immune responses to non-self (8, 9). His co-author, Ruslan Medzhitov, his student at the time, published a follow on 20 years later, showing that Janeway's pattern recognition hypothesis has subsequently been proven correct, and in addition to suggesting a general principle of innate immune recognition, provided a conceptual framework for the integration of innate and adaptive immunity(10).

The PRR are classified into five families based on protein domain homology(8). These five families consist of the TLRs, C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), and the AIM2-like receptors (ALRs) (Table 1-1).

PRR	Members	Shared domains	Receptor location
TLR	1–10 in humans, 1–9 and 11–13 mice	LRR, TIR	Cell surface, endosomal compartments
CLR	Dectin-1, Dectin-2, DC-SIGN, mannose receptor, CLECSF 8 etc	C-type lectin	Cell surface
NLR	NOD1 (NLRC1), NOD2 (NLRC2), NLRC3–5, NLRP1–9 and 11–14, NAIP1, -2, -5, -6	Nucleotide binding, LRR	Cytoplasm, plasma, and endosomal membrane associated
RLR	RIG-I, MDA5, LGP2	DExD/H helicase	Cytoplasm
ALR	AIM2, IFI16	PYRIN, HIN-200	Cytoplasm, nucleus (IFI16)

Table 1-1. Pattern recognition receptors (adapted from Brubaker et al(8)). AIM, absent in melanoma; ALR, AIM2-like receptor; CARD, caspase recruitment domain; CLR, C-type lectin receptor; IFI, IFN, γ-inducible; LGP, laboratory of genetics and physiology; LRR, leucine-rich repeat; MDA, melanoma differentiation gene; NAIP, NLR family, apoptosis inhibitory protein; NLR, nucleotide-binding oligomerization domain receptor; NLRC, NLR family CARD domain containing; NLRP, NLR family PYD domain containing; NOD, nucleotide-binding oligomerization domain; RIG-I, retinoic acid–inducible gene I; RLR, RIG-I-like receptor; TIR, Toll/IL-1 receptor/resistance; TLR, Toll-like receptor.

PRR signal via downstream adaptor proteins. Importantly, these adaptor proteins may receive signals from multiple PRR (e.g. myeloid differentiation primary response protein 88 (MyD88) acts as an adaptor protein for multiple TLR). These adaptors must interact with an upstream and downstream signalling protein(8). There are numerous adaptor proteins described as summarised in Table 1-2.

Adapter protein	Upstream receptor interaction	Downstream signalling interaction	Location
TIRAP/MyD88	TIR domain	Death domain	Cell surface, endosomal compartments
TRAM/TRIF	TIR domain	TRAF binding, RHIM domain	Cell surface, endosomal compartments
MAVS	CARD domain	Proline-rich region, TRAF binding	Mitochondrial, peroxisomal, and mitochondria-associated membranes
ASC	PYRIN	CARD domain	Cytosol, mitochondria

Table 1-2. Pattern recognition receptor downstream adaptor proteins (as adapted from Brubaker et al(8).ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase recruitment domain; MAVS, mitochondrial antiviral signalling protein; RHIM, RIP homotypic interaction motif; TIR, Toll/IL-1 receptor/resistance; TIRAP, TIR-containing adaptor protein; TRAF, TNF receptor–associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain–containing adaptor-inducing IFNβ.

1.1.1.2.3. Toll like receptors

Perhaps the best described PRR are the TLR. As they shall be important in this thesis, I shall specifically highlight their structure and pathway as an example of innate immune functioning. Toll is a receptor that was discovered in Drosophilia where it was found to be essential for establishing dorsoventral polarity during embryogenesis (11). Subsequently TLR were also found to play an important role in the immune system of insects, leading to their study in vertebrate immunology (12). TLR are essentially glycoproteins, consisting of a leucine rich repeat (LRR) and a Toll/IL1-receptor/resistance (TIR) cytoplasmic domain(Fig. 1-1)(13). The LRR forms a horse-shoe like shape which is provides a concave binding surface for the binding of various pathogen ligands. Each TLR has a specific pathogen ligand that it recognises. The cellular location of the TLR is also important in defining what it binds to and what it defines as non-self. The various TLR ligands are summarised in Table 1-3(14).

TLR	Ligand	Pathogenic origin
TLR 1	Tri-acyl lipopeptides	Bacteria and mycobacteria
TLR2	Lipoprotein, peptidoglycan, lipoteichoic acid, glycolipids, porins, atypical lipopolysaccharide(among others)	Gram positive bacteria, mycobacteria, Neisseria, fungi
TLR3	Double stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram negative bacteria, RSV
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides, lipoteichoic acid, zymosan	Mycoplasma, gram positive bacteria, fungi
TLR7	Single stranded RNA	Virus
TLR8	Single stranded RNA	Virus
TLR9	CpG containing DNA	Virus and bacteria
TLR10	Not defined	
TLR11	Not defined	

Table 1-3. TLR ligands and their origin (adapted from Akira et al(14)). TLR-Toll like receptor,RSV-respiratory syncytial virus. TLR-Toll like receptor

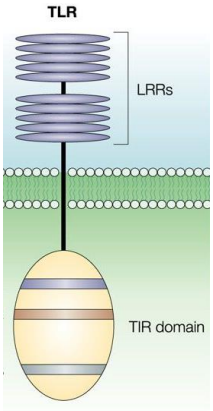


Figure 1-1. Toll like receptor (TLR) basic structure (adapted from Akira et al(14)) .TLR have a leucine rich repeat (LRR) end and an intracellular TIR(Toll/IL-1 receptor/resistance) domain.

Downstream of the various adaptor proteins, there are further complex signalling pathways, including interleukin 1 associated receptor family (IRAK), TNF receptor associated factor (TRAF) and IκB kinase (IKK) mediated phosphorylation and ubiquitination and ultimately nuclear factor-κB (NF-κB) transcription or IFN regulatory factor (IRF) activation. These pathways all differ slightly, depending on the TLR, and ultimately give rise to the transcription of specific inflammatory genes, and ultimately a specific cell phenotype in response to the particular ligand(15).

The focus of this shall be on the specific anti-viral arm of the innate immune system that has evolved to respond to viral and non viralnucleic acid ligands.

1.1.1.3. The innate antiviral immune system

There is a rapid and potent response to viral infection in the innate immune system. Viruses are particularly adept at rapid mutation and adaptation, and therefore the innate immune system has had to develop a robust non-specific response to early viral infection. Viral diseases display a sex difference in clinical phenotype, and innate response (16, 17). In addition, SLE has been described as a disease that resembles a viral infection, without any identifiable virus, in that it has a type 1 IFN transcription signature, an idea which shall be further explored later(1). For these reasons, the specific type 1 IFN antiviral innate response will be described in detail here.

1.1.1.3.1. Type 1 IFN

The type 1 IFN family is a class of cytokines, named for their ability to interfere with the replication of virus *in vivo* and *in vitro*(18). The family consists of IFNα and IFNβ as well as IFNε, IFNκ and IFNω. For the purposes of this thesis, IFNα and IFNβ will be the focus, as these have been the best described in relation to SLE. IFNα has 13 subtypes, and both IFNα and IFNβ are coded for on chromosome 9. Nearly every cell can produce type 1 IFN but, during the course of an infection, pDC are the chief producers of type 1 IFN (19).

1.1.1.3.2. Type 1 IFN signalling

All type 1 IFNs signal via the IFN receptor (IFNAR) which has 2 subunits (Fig. 1-2(20)). Each type 1 IFN subtype binds to the receptor subunits with different affinities, and different IFNAR densities exist in different tissue types, causing slightly different upregulation of IFN stimulated gene (ISG) transcription and subsequent anti-viral response depending on subtype and tissue type(21).

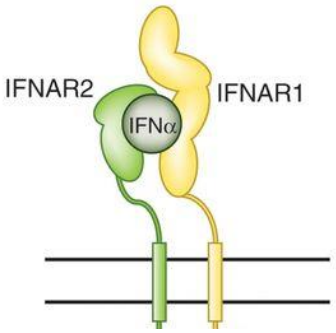


Figure 1-2. (From Randall et al(20)) The type 1 IFN receptor consist of 2 subunits. IFNAR-IFN receptor.

When type 1 IFN binds to the IFNAR, the receptor subunits are endocytosed and activate tyrosine kinases (Tyk)2 and janus kinase (Jak)1. This results in the phosphorylation of signal transducer and activator of transcription (STAT) 1 and 2, with complex formation with IRF9, resulting in the IFN stimulated gene factor 3 (ISGF3) complex. ISGF3 translocates to the nucleus, where it binds IFN stimulated regulatory elements (ISRE) in the promoter regions of ISG (Fig. 1-3(22)).

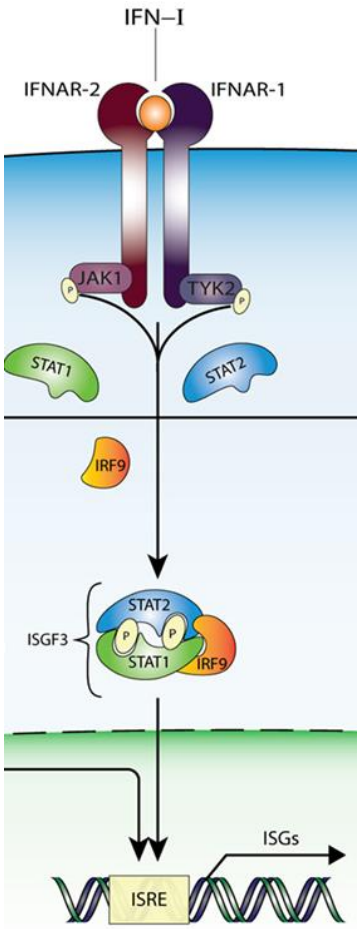


Figure 1-3. Type 1 IFN leads to transcription of ISG (From Michalska et al(22)). IFN-IFN, IFNAR-IFN receptor, JAK-janus kinase, TYK-tyrosine kinase, IRF-IFN regulation factor, STAT- signal transducer and activator of transcription, ISGF3- IFN stimulated gene factor 3, ISRE-IFN specific response element, ISG-IFN stimulated genes.

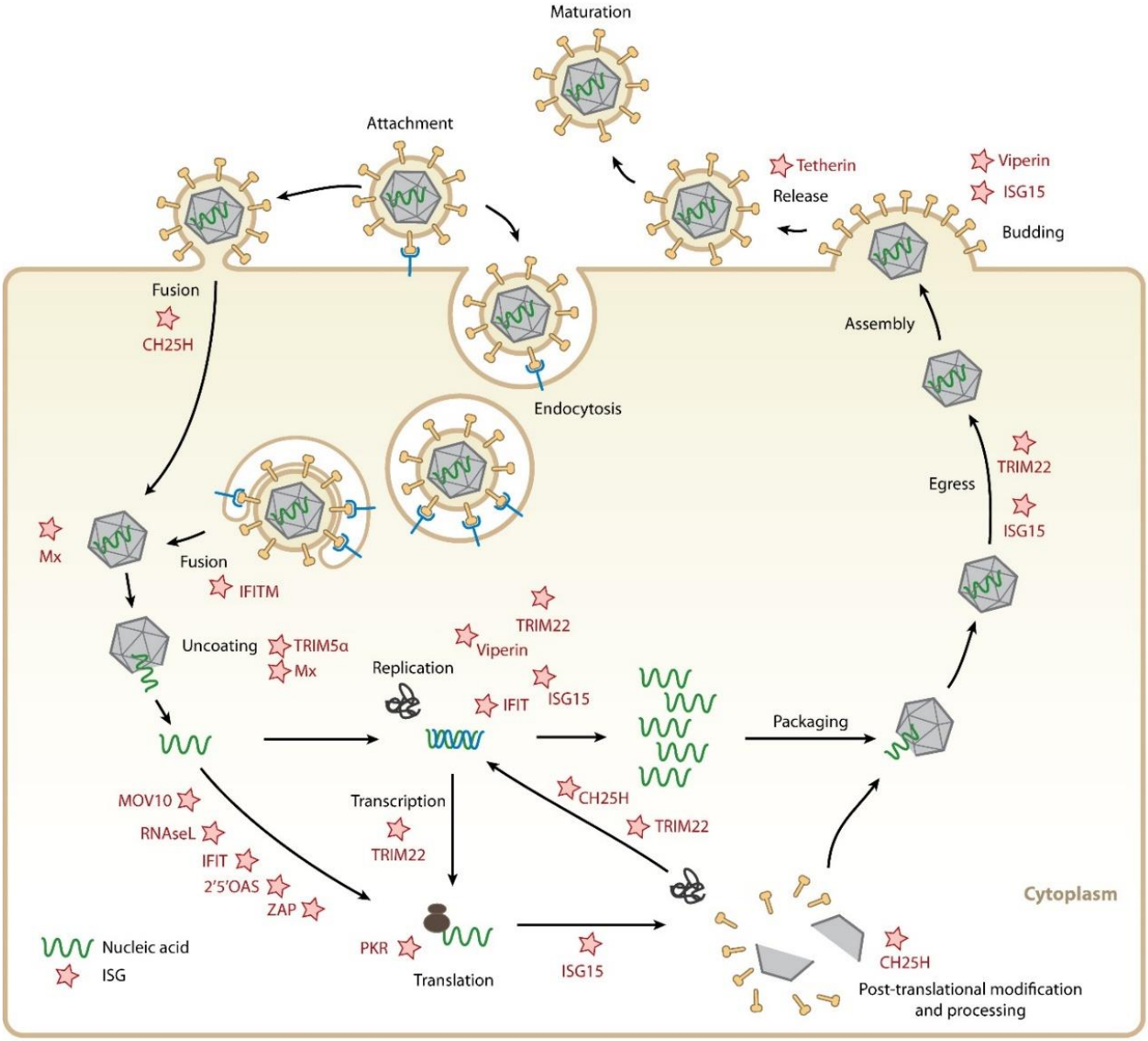
1.1.1.3.3. Type 1 IFN function and IFN inducible genes

Type 1 IFNs cause cells to behave in a typical antiviral manner. They are pro-apoptotic and cytostatic, and induce the transcription of potent viral restrictor proteins(18). Type 1 IFN has been shown to increase activation of monocytes and dendritic cells, increase chemotaxis, antigen presentation and T cell activation(23). Type 1 IFN enhances T cell survival, activation and clonal expansion, promotes B cell antibody production and class switching(23). As will be shown later, many of these type 1 IFN induced anti-viral immune functions are similar to the immune abnormalities seen in SLE. It is important to note that the type 1 IFN response may have developed to detect and respond to viral nucleic acid ligand, but is not specific to nucleic acids of viral origin.

ISG display a large variety of anti-viral functions. ISG include genes encoding for PRR such as *TLR9, RIG-1, STING, MAVS* and *cGAS* (among many others). Many ISG encoded proteins are potent antiviral effectors. These include inhibitors of viral entry (e.g. MX1, IFITM, TRIM), inhibitors of viral translocation and replication (e.g. IFIT, ISG15) and inhibitors of viral assembly and shedding (e.g. tetherin and viperin) (Fig. 1-4(24)). ISG also include transcription factors such as IRF7 and IRF3. .

Included in the ISG, are genes involved in regulation of IFN response, such as *SOCS*, a JAKSTAT inhibitor and *USP18*, which maintains long term desensitisation to IFN(25).

ISG indirectly measure for the presence and action of type 1 IFN, by measuring the genes that type 1 IFNs upregulate. ISG can be measured using a single gene, commonly *MX1*, or by measuring multiple genes, which are used to build a type 1 IFN score (26). Measuring gene expression may be complex and relatively time consuming. Very early data is emerging that the expression of the protein encoded by certain ISG (e.g. tetherin) on cell surfaces closely correlates to the gene expression and may serve as an more easily measurable proxy measure for type 1 IFN activity(27), although it is not known whether surface tetherin expression accurately correlates with ISG score.



Schneider WM, et al. 2014.
Annu. Rev. Immunol. 32:513–45

Figure 1-4. Direct anti-viral effector IFN stimulated genes (ISG) (From Schneider et al(24)). ISG are specific genes, upregulated by type I IFN which code for proteins that directly inhibit viral entry, fusion, uncoating, transcription, replication, translation and release. ISG are represented by stars in the figure above which shows the effector function of a few of the ISG.

1.1.1.3.4. Nucleic acid sensing and type 1 IFN production

Type 1 IFNs are produced when cells sense viral (or endogenous) RNA or DNA through specific cytoplasmic or endosomal nucleic acid PRR. Endosomal TLRs 3, 7 and 9 sense nucleic acids. TLR3 senses double stranded RNA (DsRNA), and signals via the adaptor protein TRIF (TIR domain–containing adaptor-inducing IFNβ) to induce type 1 IFN production.

TLR7 specifically senses single stranded RNA (ssRNA), while TLR9 senses DNA with a CpG motif. TLR7 and TLR9 are transported to appropriate intracellular compartments by UNC93B1 and require cleavage to recruit MyD88. All TLR7 and 9 signalling requires MyD88, but additional factors determine whether TLR7 or 9 engagement will result in the production of type I IFNs (IFNs) or pro-inflammatory cytokines. These factors include the mode of ligand entry and the intracellular compartment in which the TLR encounters its ligand. DNA and RNA viruses, CpG ODNs and small DNA/RNA immune complexes enter pDCs through endocytosis. If adaptor protein 3 (AP3) or microtubule-associated protein 1A/1B-light chain 3 (LC3) are recruited then the IFN-regulatory factor 7 (IRF7) endosome forms, leading to type I IFN production. Several molecules and pathways are involved in this process including IκB kinase-α (IKKα), osteopontin (OPN), SLC15A4, Bruton's tyrosine kinase (BTK), BLOC1, BLOC2, DOCK2, PACSIN1 (protein kinase C and casein kinase substrate in neurons protein), phospholipid scramblase 1 (PLSCR1), viperin, SCARB2 and the mammalian target of rapamycin (mTOR) pathway. This ultimately leads to the phosphorylation of IRF7 and transcription of type 1 IFN. Alternatively, TLR 7 and 9 containing compartments can form NF-κB endosomes, resulting in the production of pro-inflammatory cytokines and chemokines (e.g. interleukins-IL6, IL12 and tumour necrosis factor-TNF). This process requires IRF5, BTK and Solute Carrier Family 15 Member 4 (SLC15A4)(28). TLR9 signalling is represented in Fig. 1-5(28), and TLR7 signalling follows a very similar path, except that ssRNA is the ligands that acts as the trigger.

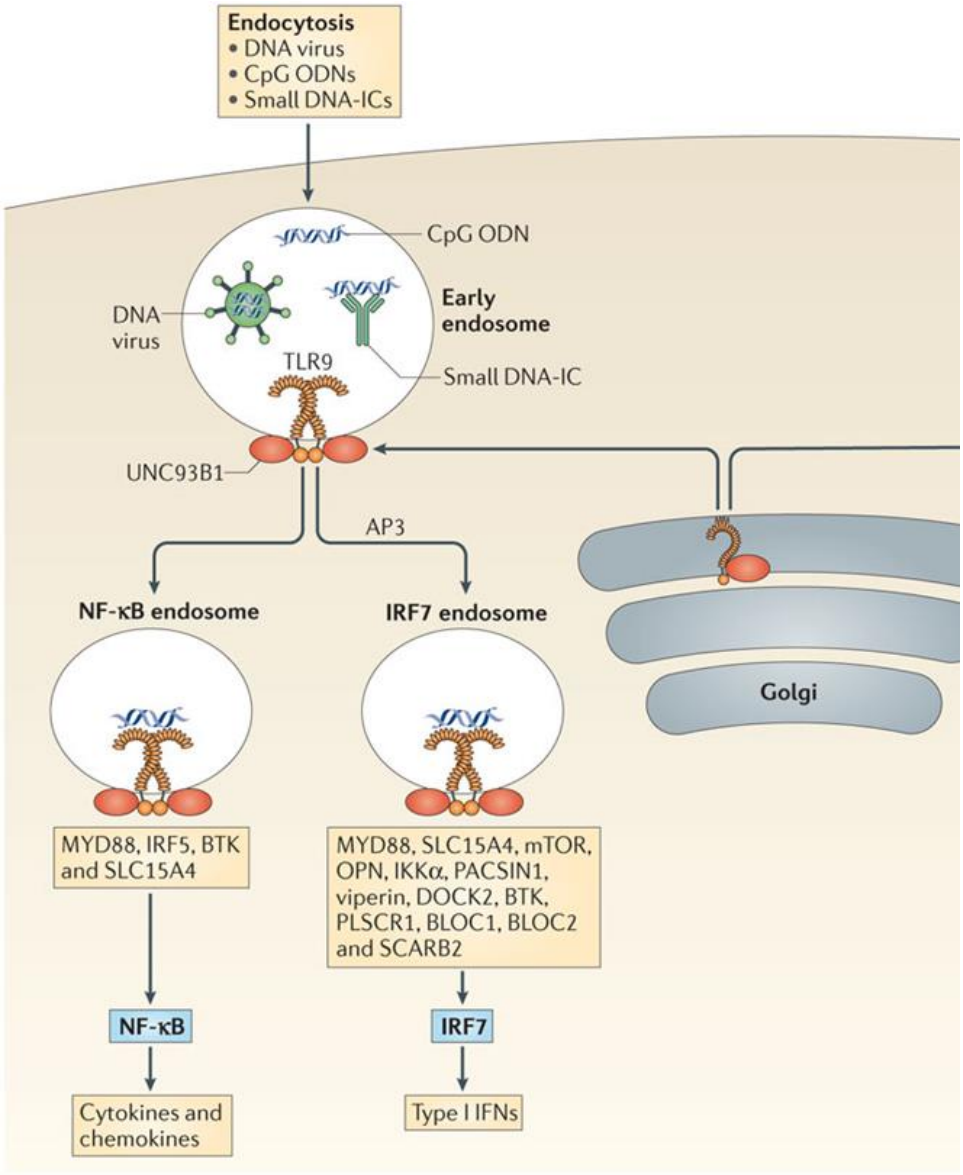


Figure 1-5. Toll like receptor (TLR)9 signalling in Plasmacytoid dendritic cells (pDC) (From Sweicki et al(28)). In TLR9 signalling, CpG containing DsDNA or a synthetic CpG ligand enter the endosome directly or via immune complex and activate TLR9 signalling. This occurs via an IRF7 or NF-κβ endosome to result in the production of type 1 IFN or other cytokines respectively. IC-immune complex, AP3-adaptor protein 3, MyD88 myeloid differentiation primary response protein 88, IRF-IFN regulatory factor, SLC 15A4-Solute Carrier Family 15 Member 4, BTK-Bruton's tyrosine kinase, PACSIN1 -protein kinase C and casein kinase substrate in neurons protein,PLSCR1- phospholipid scramblase 1, mTOR-mammalian target of rapamycin, IKKα- IκB kinase-α,OPN- osteopontin, BLOC- biogenesis of lysosome-related organelles complex, DOCK-dedicator of cytokinesis, SCARB2- scavenger receptor class B member 2.

In addition to the endosomal sensors in pDC, there are intra-cytoplasmic nucleic acid sensors capable of inducing type 1 IFN production.

DsRNA sensors are retinoic acid-inducible gene (RIG)-like receptors (RLR): RIG-1, melanoma differentiation-associated protein 5 (MDA5) and LGP2. After recognition of dsRNA, RIG-1 and MDA-5 undergo conformational changes, resulting in the exposure of caspase activation and recruitment domains (CARD) in the N-terminal. This then interacts with the CARD of the mitochondrial antiviral signalling protein (MAVS), an adaptor protein, in the outer membrane of mitochondria (29). MAVS forms aggregates in the outer membrane of mitochondria, which interact with downstream signalling proteins, IKK complex-NF-κ and TBK1/IKKi-IRF3, resulting in the expression pro-inflammatory and type I IFN genes, respectively. LGP2 is thought to regulate RIG-I- and MDA5-mediated antiviral responses (29).

Intracellular DNA is derived from viruses, bacteria, and mitochondria, and is recognized by cytoplasmic DNA PRR, including cyclic GMP-AMP synthase(cGAS), AIM2, DEAD-box helicase 41 (DDX41) and IFN alpha-inducible protein 6 (IFI16). These result in the production of type 1 IFN and other cytokines. cGAS is a newly discovered potent DNA sensor that signals via stimulator of IFN genes (STING)(30, 31).

1.1.1.3.5. Interferon regulatory factors

IRF are important in the interferon signalling pathway s of all 3 types of IFN(32). Briefly, IRF are a family of 9 factors, which are involved as transcription factors in the interferon signalling pathways. In addition, they play a role in immune cell development, regulation of immune response, oncogenesis and metabolism(32). IRF function differently in all 3 classes of IFN. In type 1 IFN via TLR 7 or 9 stimulation, IRF7 and to a lesser extent, IRF are important in the downstream transcription of type 1 IFN(33). IRF7, IRF5 and IRF3 are essential in the RIG-1/MDA5 mediated production of type 1 IFN, whereas IRF8 may play a regulatory role(33).

1.1.1.3.6. Plasmacytoid dendritic cells-brief overview

As pDC are the chief cell that produce type 1 IFN(34, 35), they will briefly be described here .In general, dendritic cells(DCs) act as a bridge between the innate and adaptive arms of the immune system. They are able to respond to molecular PAMPS and present antigen to the specific T cells in the adaptive immune system. DC are divided by the nature of the pathogen that they are primed to respond to and include the subtypes plasmacytoid DC (pDC) and conventional DC (cDC) 1 and 2(36). pDC develop from common lymphoid progenitors under the influence of E2-2, a basic haemophagocytic lymphohystiocytosis protein and the relative lack of ID2, an inhibitor of DNA binding(36). Growth factors GM-CSF (acting through STAT5)

and Flt3L (acting through STAT3) respectively inhibit or enhance pDC development by modulating the expression of ID2 and E2-2. .pDC specialise in the innate anti-viral response by producing type 1 IFN after the sensing of cytoplasmic or endosomal nucleic acid(28). Human pDCs do not express the lineage-associated markers CD3, CD19, CD14, CD16 and CD11c(37). They are characterised by the expression of BDCA2 (also known as CLEC4C), CD123 and the immunoglobulin superfamily receptor immunoglobulin-like transcript 7 (ILT7; also known as LILRA4)(37). Although most cells can produce type 1 IFN after TLR7 and TLR9 stimulation, pDC are known to produce by far the most, in the order of 1000 times more than any other cell type. Upon stimulation with TLR7 or TLR9 ligands, pDC downregulate their expression of BDCA2(38).

1.1.1.4. Summary

In this brief overview, the overall scheme and functioning of the highly complex immune system has been described, with a specific focus on the type 1 IFN related, innate, anti-viral immune system, which has a relevance to sex and jSLE. In the next section, the concepts of sex and puberty will be described and the limited data about their role in immune development and functioning will be summarised.

1.1.2. Sex

Sex is a biological construct defined by chromosomes, sex organs and hormones. Gender is defined by behaviours and activities influenced by society and culture. Sex is an important and neglected biological variable, especially within the immune system. Unfortunately, immunology ranks among the lowest of the scientific disciplines for the reporting of sex based differences(39). There was a perception in early research that female animals introduced monthly variability into data and therefore many preclinical studies included male animals only(40). The Food and Drug Administration in the 1970s recommended that females of reproductive age be excluded from clinical studies, and therefore females have been historically under-represented in pre-clinical and clinical research(3). Sex is an important biological variable that may provide insights into disease pathogenesis, especially in diseases with a strong sex bias, such as SLE. Recently the National Institute of Health and other Canadian and European agencies have issued a call for sex differences to be represented in clinical and pre-clinical studies(3). A brief summary of the literature regarding the role of sex in the immune system is given below.

1.1.2.1. Sex and the immune system

Sex, as a biological variable influences the immune system in anatomical and physiological terms, in the transmission of, exposure to, clearance of and subsequent immunity to, micro-organisms. The psychosocial variable, gender, may influence the immune system in that it

influences societal behaviours, environmental exposures and health seeking. For the purpose of this thesis, sex will be referred to as a biological variable, with the recognition that gender also influences immunity, although not discussed further here(3).

1.1.2.1.1. Sex and disease

As mentioned before, disease states often highlight underlying physiological processes. Many diseases display a sex bias in terms of incidence and prevalence as well as severity and outcome. A non-exhaustive summary is provided in Fig. 1-6 from an excellent review on the subject by Klein et al(3). The general trend that emerges is that females seem to mount an enhanced immune response to most pathogens when compared to males. This offers females relative protection from infection and some cancers, while increasing their risk of autoimmunity and morbidity in viral disease where a hyper-active response may be detrimental (e.g. HIV, influenza).

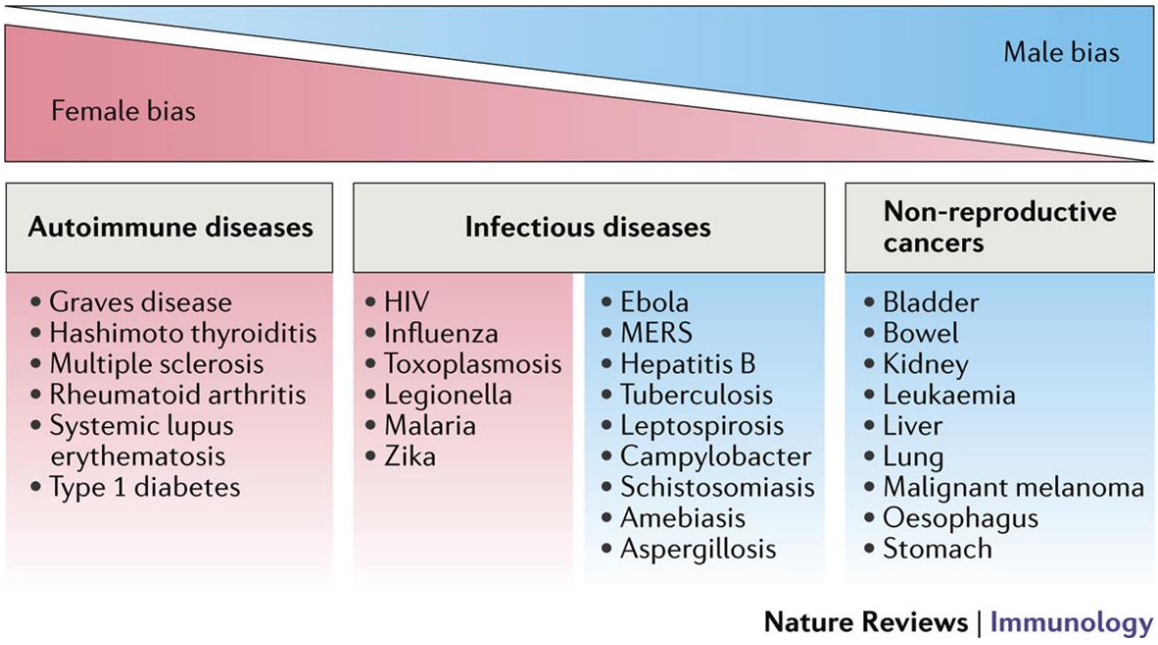


Figure 1-6. Summary of sex differences in disease manifestations (From Klein et al(3)).
Generally, females show an increased susceptibility to autoimmune disease whereas males bias toward non-reproductive cancers. There are also differences in susceptibility to infectious diseases. HIV-human immunodeficiency virus, MERS-middle east respiratory syndrome.

It appears that these sex differences in disease occur at all ages. New-born males have a higher rate of death from infection than new-born females(41). In developing countries, male children have higher rates of malaria, trematode and nematode infections(42). In adults, human immunodeficiency virus (HIV) infected women have a higher CD8+ T cell activation, when adjusted for viral load, and less circulating viral RNA than men. Despite this, women have a 1.6 fold higher risk of progressing to advanced immune deficiency syndrome(AIDS) at the

same viral load as men(43). Prevalence of infection with influenza virus is higher in men, but death following influenza infection is higher in women. Tuberculosis and Cryptococcus infection are more common in men, along with hepatitis B infection(3). Men are more likely to die of sepsis than females(44).

It is well established that females are more prone to develop almost all types of autoimmune disease. The exception to this rule (which is not included in Fig 1-6) is HLAB27 mediated disease such as ankylosing spondylitis and juvenile onset enthesitis related arthritis, which are more common in males after puberty.

Sex differences have also been described in vaccine response (45, 46) (Figure 1-7). Consistently, females mount a 'stronger' response than males, achieving higher titres of antibody post-vaccination. In addition, it has been shown that females are more likely to suffer adverse events secondary to immunisation than men (i.e. vaccine side effect), but less likely to contract the disease in the future (i.e. vaccine failure)(46). Indeed, in dose response studies in influenza vaccine, women who received half the dose of inactivated influenza vaccine than men achieved equivalent protective antibody titres(47).

Target group	Vaccine	Sex difference in Immune response	Sex difference in adverse reactions	Age (years)
Children	Hepatitis B	Greater in females	Not defined	<12
	Diphtheria	Greater in females	Not defined	<2
	Pertussis	Greater in females	Not defined	<2
	Pneumococcal	Greater in females	Not defined	6–9
	Rabies	Greater in females	Not defined	6–9
	Measles	Greater in females or equivalent in both sexes	Increased in females	<3
	RTS,S vaccine against malaria	Greater in females	Increased in females	<2
	Human papillomavirus	Greater in females	Increased in females	5–17
Adults	Influenza	Greater in females	Increased in females	18–49
	Hepatitis B	Greater in females	Increased in females	>18
	Herpes virus	Greater in females	Not defined	>18
	Yellow fever	Greater in females	Increased in females	>18
	Rabies	Greater in females	Not defined	>18
	Smallpox	Greater in females	Not defined	>18
Aged adults	Influenza	Greater in females	Increased in females	>65
	Td/Tdap	Greater in males	Increased in females	>65
	Pneumococcal	Greater in males	Increased in females	>65
	Shingles	Not defined	Increased in females	>65

Figure 1-7. Sex differences in response to vaccination (From Klein et al(3)). Td/Tdap-tetanus, diphtheria and pertussis

Cancers occurring outside of the reproductive tract show a bias towards males (48). Males have double the risk of mortality than females from all types of malignancy(49). Immune based cancer therapies, such as programmed cell death 1 ligand 1 specific monoclonal antibodies have been reported to be more effective in females than males with malignant melanoma (3, 50). Therefore, an enhanced immune response in females may offer better immune surveillance of tumour cells.

In summary, sex differences in disease phenotypes generally seem to indicate that females at all ages have an 'enhanced' immune reaction to infection than males.

1.1.2.1.2. Sex and the adaptive immune system

Compared to the overall volume of immunology research, research that includes sex as a variable in immunology is rare. Despite this, from the research that is available, it is clear that sex affects most arms of the immune system, as will be summarised here. The general trend

mirrors the findings in disease phenotype, in that females tend to produce an enhanced or exaggerated response as compared to men.

From the earliest screening of self-reactive T cells in thymic tissue by the autoimmune regulator (AIRE), sex differences are present in the immune system. *AIRE* gene expression has been shown to be decreased in female thymic tissue in two separate recent studies (51, 52). Dragin and colleagues associated diminished *AIRE* expression with oestrogen-associated gene methylation, while Zhu and colleagues demonstrated that the androgen receptor bound the *AIRE* promoter region and increased *AIRE* expression. Both demonstrated that *AIRE* expression affected thymic expression of tissue-specific antigens and, using murine models of multiple sclerosis and thyroiditis, found that altering sex steroid production influenced susceptibility to autoimmunity in an AIRE-dependent manner(53).

CD4+ T cells have been widely shown to be increased in healthy female adults and children, when compared to males (54-56). Interestingly, even in the context of HIV infection, which depletes CD4 T cells, female new-borns, children and adults have been reported to have an increased percentage of CD4 T cells in peripheral blood mononuclear cells (PBMC) when compared to males (57-59). Female T cells have been shown to have an improved cytotoxic activity and upregulation of inflammatory genes, and almost half of the activated genes in human T cells have an oestrogen response element in their promoter region(60). Evidence suggests that males have more T-regulatory cells (Treg) than females(61).B cells in females have been shown to be increased in number and produce more immunoglobulin than in men(54, 62). The described sex differences in the immune system are summarised below in Table 1-4.

	Female	Male
CD4 T cells	↑CD4 number ↑activation ↑IFNɣ	↑IL17 production
CD8 T cells	↑cytotoxic activity ↑antiviral gene transcription(RIG, IFNG, IFI6)	
Treg cells		↑Treg numbers
B cells	↑B cell number ↑Basal levels Immunoglobulin ↑ antibody responses	
Antigen presenting cells	↑phagocytic activity ↑antigen presentation	Neutrophils-↑TLR4 and TNF

Table 1-4. Reported sex differences in the immune system (from Klein et al) (3). IFN-IFN, Treg-T regulatory cell, TLR-toll like receptor, TNF-tumour necrosis factor, Il-interleukin.

1.1.2.1.3. Sex and the innate immune system

There is very little data that investigates sex differences in the innate immune system. From what exists, it seems that males seem better primed immunologically to respond to bacterial PAMPs. PBMC from human males produce more tumour necrosis factor alpha (TNFα) after lipopolysaccharide (LPS) stimulation than females (63). Male neutrophils have been shown to express more TLR4 than females (64, 65). Recent data from the Human Functional Genomics Project, a large project that assessed the variability of human cytokine responses to a large panel of microbial and metabolic stimuli in a group of 500 healthy volunteers; female sex was associated with higher circulating levels of IL-1Ra, lower IL-18 binding protein (IL-18BP), and did not associate with IL-6(66, 67). Spontaneous neutropil apoptosis has been shown to be delayed in women compared to men(67).

This is not the case in the type 1 IFN, anti-viral innate immune system, where there is good evidence that females have a more robust response than males as I will demonstrate in detail later(16, 68, 69).

1.1.2.2. Summary

The difference in immune disease phenotype between sexes highlights the importance of sex as a variable when studying the immune system. The data are relatively scarce, but indicate that females, in general, display an enhanced immune response when compared to males. Males and females differ by sex chromosome and sex hormone. These concepts will now be described and what is known about how they associate with the immune system will be summarised.

1.1.3. Sex Chromosomes

Females have two X chromosome and males have a single X and Y chromosome. Females however express genes from only one of their X chromosomes. X gene dosage compensation is achieved, at least partially, through random X chromosome inactivation(XCI) (70). The choice of which chromosome is silenced occurs early in cell development and randomly results in an inactivated X (Xi) and an active X (Xa). The Xi has an exclusion of RNA polymerase II and transcription factors as well as epigenetic modifications to repress the Xi(71). This leads to a natural mosaicism in females, where cells express either a maternal or paternal derived Xa chromosome. This natural mosaicism protects against the effects of X-linked deleterious deletions and provides an added diversity in the immune response(72). XCI relies on the expression of X-inactive-specific-transcript (Xist), a long, non-coding RNA that is essential for female survival(73). Xist is mono-allelically upregulated on an X chromosome and triggers that X to become transcriptionally silenced(73).

Up to 15% of Xi genes in female humans escape XCI (74) with wide variation between gene loci and individuals. These regions are known as pseudo-autosomal genes(75).

1.1.3.1. X chromosome related immune disease

As mentioned before, immune associated disease often directs our understanding of the functioning of the immune system. An indication of the importance of the X chromosome in immunity is the clinical manifestation of X-linked immune disorders which will be briefly summarised here. The prototypic hereditary autoimmune disease, immune-dysregulation, poly-endocrinopathy, enteropathy, X linked syndrome (IPEX) is among these and results from a mutation in *FOXP3* and a subsequent deficiency or absence of T-regs, manifesting as early onset life-threatening autoimmunity. Bianchi et al (76) have compiled a table (Table 1-5) of the other X linked immune diseases, which manifest mostly as immune deficiency, but may have autoimmune manifestations. This serves to highlight how the X chromosome has an abundance of genes encoding for immune function which will be discussed in the next section.

Table 1-5. Summary of X linked immune deficiency syndromes as per Bianchi et al (76). IBD-inflammatory bowel disease, HA- haemolytic anaemia, DLE-discoid lupus erythematosus, SLE-systemic lupus erythematosus, ITP-idiopathic thrombocytopenic purpura

Disease	**Gene**	**X chromosome location**	**Major autoimmune features**
X-linked hyper-IgM syndrome (HIGM)	*CD40L*	Xq26	Sero-negative arthritis, hypothyroidism, hepatitis, IBD
X linked agammaglobulinemia (XLA)	*BTK*	Xq21.3-q22	Arthritis, Diabetes, HA, Scleroderma, Alopecia
Immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX)	*FOXP3*	Xp11.23-q13.3	Serum autoantibodies against different organs including gut, liver, skin, pancreases, kidney.
Wiskott-Aldrich syndrome (WAS)	*WAS*	Xp11.23-p11.22	Vasculitis, uveitis, renal disease, Henoch-Schönlein–like purpura, IBD, cerebral vasculitis, neutropenia, dermatomyositis, recurrent angioedema
X-linked neutropenia and myelodysplasia	*WAS*	Xp11.23-p11.22	Severe neutropenia
Chronic Granulomatous Disease	*CYBB*	Xp21.1	Sarcoidosis, Crohn's disease, Rheumatoid Arthritis, DLE, SLE, ITP, Kawasaki disease, Behchet syndrome
X linked severe combined immune deficiency (SCIDX1)	*IL2RG*	Xq13	Skin Rash, HA
Anhidrotic ectodermal dysplasia with immune deficiency NEMO/IKKγ deficiency	*IKBKG*	Xq28	IBD, Arthritis, Autoimmune HA
X linked lymphoproliferative syndrome	*SHD2D1A (1)* *XIAP (2)*	Xq25 Xq25	Hemophagocytic Syndrome, Necrotizing Vasculitis, Nephritis

1.1.3.2. X chromosome related immune genes.

The X chromosome codes for about 1200 genes, including a remarkable number of immune associated genes (Fig. 1-8(77)). The dogma is that females express only 1 copy of most of the X chromosome encoded genes, as the Xi is 'switched off'. In the immune system, data is emerging which shows that this may not always be true. Given the large number of immune encoded genes on the X chromosome, it is a reasonable extension, that if females were to express more of these genes than males, it may explain some of the sex differences seen in immune disease phenotype and immune function.

It was recently shown that in human B and T cells, there is a lack of co-localisation of Xist RNA to the Xi as seen in other somatic cells (78). In addition, the Xi in naïve B and T cells, lacks the epigenetic histone modifications present in somatic cells. This paper described the Xi as acting in a euchromatic manner in human lymphocytes. They show that human female B cells contain regions along the X that are expressed at higher levels when compared with male cells, and that these regions contain immunity-related genes (e.g. *CXCR3* and *FOXP3*)(78). Using a sequencing technique to map open chromatin sites, predicted transcription factor finding, and nucleosome position, or 'personal regulome' mapping, Qu et al found that sex was the largest influence on the variability of the 'regulome' of CD4 T cells, with a majority of the variability in regulatory elements mapping to the X chromosome(79) .

Therefore, there is emerging evidence that there may be variable inactivation and regulation of the Xi in immune cells specifically and subsequent bi-allelic expression of X encoded immune genes in females.

In addition, there should be a steady and predetermined mosaicism of paternal and maternal Xa expression in tissues in females(77). In acute inflammation or challenge, there may be proliferation of a certain subset of cells, resulting in skewing of the natural mosaicism of X chromosome expressed genes. In addition, there is an increased skewing of blood and immune cells with age in women, which may lead to an over-expression of certain X-linked alleles(80).

Therefore, the dogma that only one copy of an immune gene is always expressed in females is being challenged. In addition, the natural heterogeneity enjoyed by females through mosaicism may not be constant and may skew under various conditions resulting in over-expression of deleterious alleles. This may explain why females may have an enhanced immune response, if they have bi-allelic expression of certain X linked immune genes.

Cytokine and toll-like receptor (TLR) signaling
IL1RAPL1 and IL1RAPL2: members of the interleukin-1 receptor family
IL2RG: receptor-γ for IL-2, 4, 7, 9, 15, and 21
IL13RA1 and IL13RA2: decoy receptors for IL-13
CXCR3: chemokine receptor for CXCL 9, 10, and 11
TLR7: toll-like receptor 7
TLR8: toll-like receptor 8
BGN: endogenous ligand for TLR2 and TLR4
Bruton's kinase: TLR signaling
Interleukin-1 receptor-associated kinase 1: TLR signaling
NF-kappaB and MAPK signaling
IKBKG(NEMO): inhibitor of kappa B kinase gamma
NKRF: silencing of IFNB through NF-κB inhibition
NKAP: NF-kappaB activating protein
EDA and EDA2R: ectodysplasin A and its receptor, NF-κB, and JNK regulation
MKP4: inactivation of MAP kinases
CNKSR2: MAPK activation
Apoptosis, redox balance, and metabolism
XIAP (BIRC4): direct inhibition of caspase 3 and 7
AIFM1: apoptosis-inducing factor *via* mitochondria
IGBP1: apoptosis inhibition *via* BCR and CD79
Glucose-6-phosphate dehydrogenase: oxidative burst, ROS production, and antioxidant defense (*via* GSH)
NOX1 and NOX2: catalytic units of NADPH oxidases producing superoxide anion and ROS
Other immuno-modulators
CD40 ligand: antigen presentation and T cell activation
FOXP3: differentiation of regulatory T cells
MTCP1: T cell proliferation
VSIG4: macrophage phagocytosis and T cell inhibition
BMX: growth and differentiation of hematopoietic cells
TIMP1: wound repair and tissue inflammation
GATA1: differentiation of erythrocytes and megakaryocytes
FGF16: promotes fibroblast in tissue repair and inflammation
GAB3: macrophage differentiation
TSC22D3: anti-inflammatory and immunosuppressive glucocorticoid receptor
PFC: alternative complement pathway regulation
WAS: immune activation, BM function, cytoskeleton (Wiskott–Aldrich protein)
SH2D1A/SAP: B and T cell stimulation
ARHGAP4 and ARHGAP6: early immune cell activation *via* Rho GTPase
DUSP21: anti-inflammatory
ARHGEF9: cell cycle regulation

Figure 1-8. Genes on the X chromosome encoding proteins with immune function. (From Spolarics et al(77))

Most studies exploring the reasons for sex difference in immune diseases such as SLE, focus on the hormonal differences between males and females. X chromosome number and the expression of X chromosome associated genes is an important and neglected variable in the

study of the immune system. Part of the reason for this, is the difficulty separating out the individual contribution of X chromosome number and sex hormone environment in sexually mature, human adult volunteers, as they are inherently linked and naturally confound each other. This has been possible in mouse models as will be shown later, but not in humans. In this project, unique human volunteers have been recruited to be able to investigate the effects of X chromosome number and sex hormone individually.

1.1.3.3. Turner's syndrome

Turner's syndrome (TUS) is a disorder of the X chromosome. It is one of the commonest chromosomal disorders and affects up to 1in 2500 female infants(81). TUS describes a clinical phenotype in females, with associated complete or partial absence of the second X chromosome, with or without cell line mosaicism(82). Young people with TUS may present with lymphoedema, typical dysmorphic features, short stature, recurrent otitis media, congenital heart defects (bicuspid aortic valve and aortic coarctation), primary amenorrhoea, delayed puberty and infertility among other clinical presentations(83).

Approximately half of TUS manifests as a true 45 X aneuploidy. It may also be due to structural abnormalities in the second X chromosome. These include an isochrome of the long arms of the second X chromosome (46, X,i(X)q), which essentially manifests as an X short arm monosomy. In addition, portions of the long or short arm may be missing (Xq or Xp). A ring chromosome X (rX) may form if part of the end of both the short and long arms are missing- functionally similar to an Xp deletion(84). Any of these forms may exist with various levels of mosaicism. In addition, structural anomalies can exist in mosaic form alongside karyotypes 45,X or 46,XX(83). More complex karyotypes include chromosomal translocations and 45,X/47,XXX mosaics(83).

Essentially however, whatever the genotype, the phenotype is produced when insufficient X chromosome material is available(82). These young patients are treated individually based on their particular disease manifestation and may require growth promotion with growth hormone therapy and pubertal induction with oestrogen therapy.

Young females with TUS may provide an interesting model therefore of a phenotype with effectively one X chromosome but no Y chromosome, and potentially normal female hormone environment if treated with exogenous oestradiol.

1.1.3.4. Summary

The X chromosome is associated with immune-based disease and harbours many immune genes. Data is emerging that the X chromosome, or portions thereof, may escape inactivation in some immune cells. It is a difficult to study this in humans, as the number of X

chromosomes present corresponds to the sex hormone environment and these confound each other. Young females with TUS may provide a model which enables us to explore this further. In the next section, the effect of sex hormones, and what is known about their association with the immune system will be explored.

1.1.4. Hormones

Hormones are signalling molecules, classified as proteins, steroid hormones, catecholamines and iodothyronines.

Protein hormones are synthesised as pre-hormones and stored in secretory vesicles. They are regulated at the level of secretion and synthesis and often circulate unbound in plasma. They are hydrophilic and signal via transmembrane receptors. The gonadotrophins, luteinising hormone (LH) and follicle stimulating hormone (FSH) are examples of protein hormones, secreted by the hypothalamus.

Steroid hormones are derivatives of cholesterol that are synthesised in the adrenal gland (corticosteroids), testes or ovaries (sex hormones) or placenta. These all have closely related structures based on a cycloperhydropentano-phenanthrene four ring structure (Fig 1-9). During steroidogenesis, steroid hormones undergo various enzyme mediated modifications, specific to certain tissue/organ sites, in order to produce a cholesterol derivative that is unique to a specific receptor (Table 1-6, Figure 1-9)(85). These steroid hormones are then classified into certain subgroups i.e. mineralocorticoid, glucocorticoid, progestin, androgen and oestrogen based on site of manufacture and receptor.

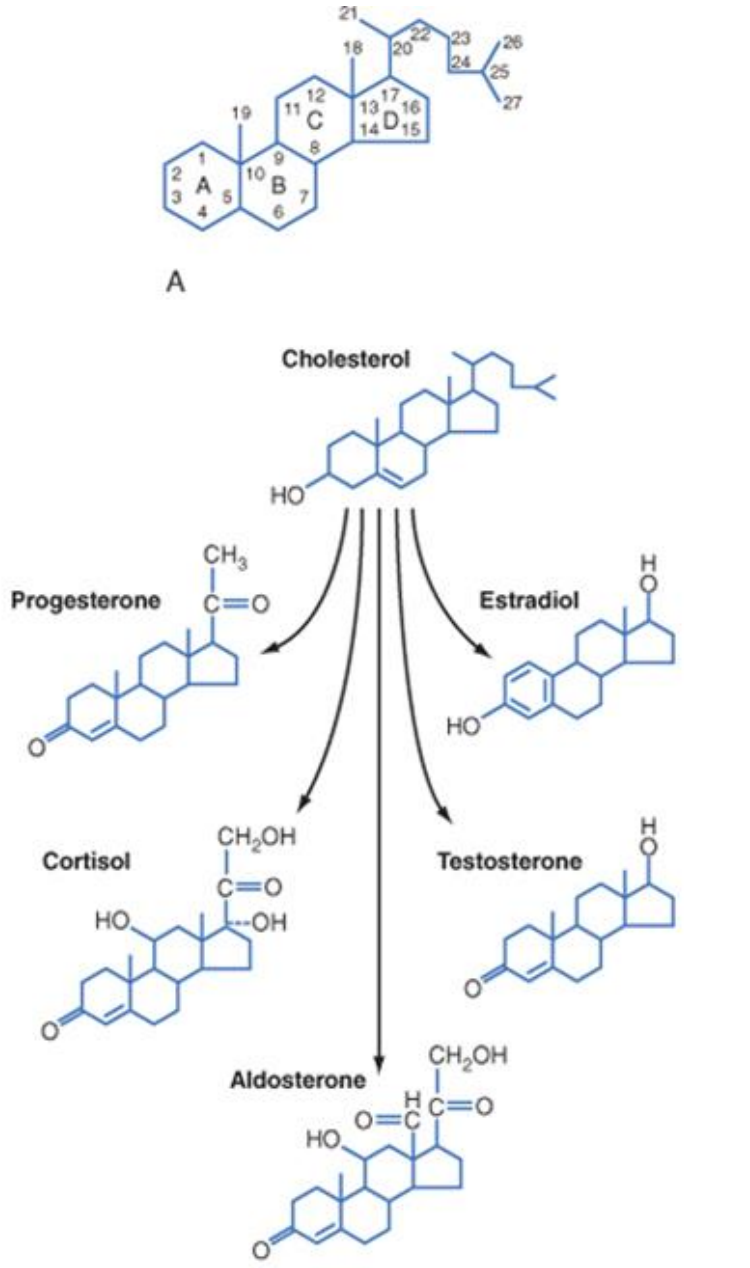
Hormones potently regulate many functions within a cell, including growth, protein expression, cell death, genetic and epigenetic changes and many more. Hormone function is not ubiquitous on all cell types however, and only certain cell types express receptors for certain hormones.

More specifically, receptors to sex steroid hormones are nuclear hormone receptors. These have the ability to enhance or inhibit the rate of expression of various genes, depending on the cell type. These receptors contain a hormone response element which acts as a DNA binding port, directly before the promoter region of gene that the hormone is acting on to enhance transcription.

Typically, oestradiol is essential for the manifestation of female secondary sexual characteristics, whereas testosterone is the male sex steroid. Progesterone plays a role in maintaining pregnancy and the menstrual cycle in females, which will be further elaborated below.

Family	No. of carbons	Hormone	Primary site of synthesis	Primary receptor
Progestin	21	Progesterone	Ovary/placenta	Progesterone receptor
Glucocorticoid	21	Cortisol, Corticosterone	Adrenal Cortex	Glucocorticoid receptor
Mineralocorticoid	21	Aldosterone, 11-deoxycorticosterone	Adrenal Cortex	Mineralocorticoid receptor
Androgen	19	Testosterone, dihydrotestosterone	Testis	Androgen receptor
Oestrogen	18	Oestradiol, Oestrone	Ovary/placenta	Oestrogen receptor

Table 1-6. Steroid hormone family (From Porterfield, Endocrine and Reproductive Physiology (85))



B

Figure 1-9. Steroidogenesis pathway (from Koeppen and Stanton 2008 (86))A. Steroid hormones all have closely related structures based on a cycloperhydropentano-phenanthrene four ring structure. B.During steroidogenesis, steroid hormones undergo various enzyme mediated modifications, specific to certain tissue/organ sites, in order to produce unique steroid hormones.

1.1.4.1. Hormones and the immune system

1.1.4.1.1. Oestradiol

Oestradiol has long been the hormone of choice when attributing blame for the increased immune response in females. It is the obvious choice as it is the hormone that is most associated with the female sex. It may not however be solely responsible. Below is a brief summary of the known effects of oestradiol on the immune system.

Many immune cells have been shown to express oestrogen receptors alpha and beta(87). In terms of the adaptive immune system, the effect of oestradiol seems to be dose dependant. Generally, low doses of oestradiol correspond with a Th1 type responses and cell mediated immunity, whereas higher doses of oestradiol, as one would expect in pregnancy, correspond to Th2 type responses and humoral immunity (3, 88). Oestradiol has been shown to modulate CD4 T cells and CD8 T cells(89). Oestradiol has been shown to bind to oestrogen response elements (ERE) in the IFNγ promoter region(90), promote Treg cell expansion in vitro and in vivo(91) and decrease TH17 IL17 production(92). Oestradiol has also been associated with increased antibody production, somatic hyper-mutation and class switching (93).

Oestradiol treatment has been shown to increase the number of neutrophils in humans(94). Oestradiol at low dose is associated with enhanced monocyte/macrophage cytokine production, but at high doses associates with suppression of these responses(95).

1.1.4.1.2. Testosterone

Testosterone is generally thought to have immune modulating effects although the data is scarce(3). Hypo-androgenism associates with increased inflammatory cytokines, antibody titres and CD4/CD8 ratios(3). Gonadotropin releasing hormone (GnRH) antagonism in men lowers testosterone levels and associates with a decrease in Treg cells, and an increase in natural killer (NK) cells(96).

1.1.4.2. Summary

Little data exists regarding the direct role of hormones in the immune system, although oestradiol is thought to have a dose dependant effect and testosterone is thought to be immune modulatory. A direct extension of this would be to examine if the immune system associated with puberty, where there is a massive change in the sex hormone environment. Puberty and its associated physical and physiological changes will be discussed below.

1.1.5. Puberty

Puberty is characterized by the maturation of gametogenesis, secretion of gonadal hormones, and the development of secondary sexual characteristics and reproductive functions. It is a period of biological change, starting on average at 11 years old in girls and 12 years old in boys,

and lasting approximately 4 years, with a wide variation in the normal age of onset and duration(97). Adolescents are defined by the WHO as young people between the ages of 10-19(98). The term 'adolescence' is used widely as a synonymous term for puberty, but has a wider age range and additional non-biological connotations of cognitive, psychological, and social change(99). Adolescents are under-represented in clinical and pre-clinical research which focuses either on children or adults. A contributing reason for this may be the great variation inherent in this time of change, which involves a cataclysmic shift in the hormonal and social functioning of young people. Another reason is the lack of adolescent focussed healthcare, with artificial barriers in healthcare between paediatric and adult services. Adolescents are therefore an under-utilised source of new information, especially in terms of how sex hormones affect the immune system.

Puberty consists of a series of predictable events that show some variation in timing, onset and sequence. In females, these consist of breast development, and the enlargement of the internal female organs with the achievement of menstruation (menarche). In males, puberty consists of the enlargement of the genitalia and an increase in testicular volume along with the breaking of the voice. Both sexes undergo thelarche (development of pubic and axillary hair) and a growth spurt. (100)

Pubertal stages were classified in a widely used classification system by Tanner and Marshall in 1969 (101).

The problems with Tanner based staging was that it is quite complex, with 5 different stages, that are too descriptive for routine screening of pubertal status. In addition, Tanner staging is based on physical phenotype, and requires physical examination. Ethically and practically, it is difficult to justify performing physical examination of breasts and genitalia on young people routinely. In research involving pubertal development, it is not feasible to perform full Tanner staging on large cohorts of healthy young volunteers.

Therefore, more recently, a simpler method of classifying pubertal phase (to replace stage) has been developed to identify young people as either pre-puberty, in puberty or post-puberty(102). This divides the pubertal phases into 3 simple, easily definable phases, namely, pre-puberty, in puberty and completing puberty. In addition, this system uses self-reportable criteria, such as menarche, voice deepening and hair growth, to make it more feasible and acceptable to young people. The Royal College of Paediatrics and Child Health (RCPCH) has adopted these three pubertal phases in the Childhood and Puberty close monitoring chart, shown in Table 1-7 and 1-8 below.

Pre-puberty (Tanner stage 1)	In puberty (Tanner stage 2-3)	Completed puberty (Tanner stages 4-5)
If **both** of the following:	If **any** of the following:	If **any** of the following
No signs of pubertal development High voice	Early pubic or armpit hair growth Enlargement of testes or penis	Voice fully broken Moustache and early facial hair growth Adult size of penis and axillary hair

Table 1-7. Male pubertal phases (RCPCH Growth Chart(103))

Pre-puberty (Tanner stage 1)	In puberty (Tanner stage 2-3)	Completed puberty (Tanner stages 4-5)
If:	If **any** of the following:	If **all** of the following
No signs of pubertal development	Any breast enlargement so long as nipples enlarged Any pubic or axillary hair	Starting periods (menarche) with breast, pubic and axillary hair development.

Table 1-8. Female pubertal phases (RCPCH Growth Chart(103)).

There is considerable variation in the timing of puberty, but it usually follows a specific sequence. In females, it starts with breast development, followed by pubic and axillary hair development and culminates with menstruation. In males, the onset is marked with testicular and genital enlargement, followed by pubic hair and axillary development. In addition to the variation in the timing of onset, there is a variation in the transit between each stage, although there is roughly a year between each Tanner stage(100).

In the UK, premature puberty in females is defined as the development of secondary sexual characteristics before the age of 8 in females and 9 in males. Delayed puberty is defined as the absence of secondary sexual characteristics by 13 years in girls, and 14 years in boys(100). A study of 1166 females in the UK showed the median self-reported onset of menarche to be 12 years and 11 months(104).

1.1.5.1. Hormonal changes in puberty

Puberty is initiated by the increase in the pulsatile release of GnRH by the hypothalamus. There has been a lot of investigation into the trigger of this event, which is associated with genetic, ethnic and social associations, although the exact trigger is unknown (105, 106). The increased pulsatile nature of GnRH release leads to an increase in the release of FSH and LH. FSH causes maturation of ovarian follicles and release of oestradiol. This, in turn promotes

breast development, bone growth and maturation, and the eventual achievement of menstruation (Fig. 1-10). A mature menstrual cycle consists of a follicular phase, with the maturation of a follicle, under the influence of FSH. A mid cycle surge of LH triggers ovulation. In the luteal phase, the corpus luteum supports the endometrium with the release of progesterone and oestradiol, which eventually decreases in the absence of an implanted embryo, resulting in menstruation.

Male puberty is also initiated by GnRH pulses, leading to an increase in LH, which stimulates the Leydig cells of the testes to produce testosterone. This stimulates the growth of the seminiferous tubules and increases testicular volume. Testosterone also increases the size of the genitalia, deepens the voice, and stimulates growth of hair and muscular development. FSH stimulates the growth of the seminiferous tubules, but also stimulates the release of inhibin b which inhibits further FSH. There is a peripheral conversion of some testosterone to oestradiol, resulting in growth, skeletal maturation and some breast development which may occur in males during puberty (Fig. 1-11).

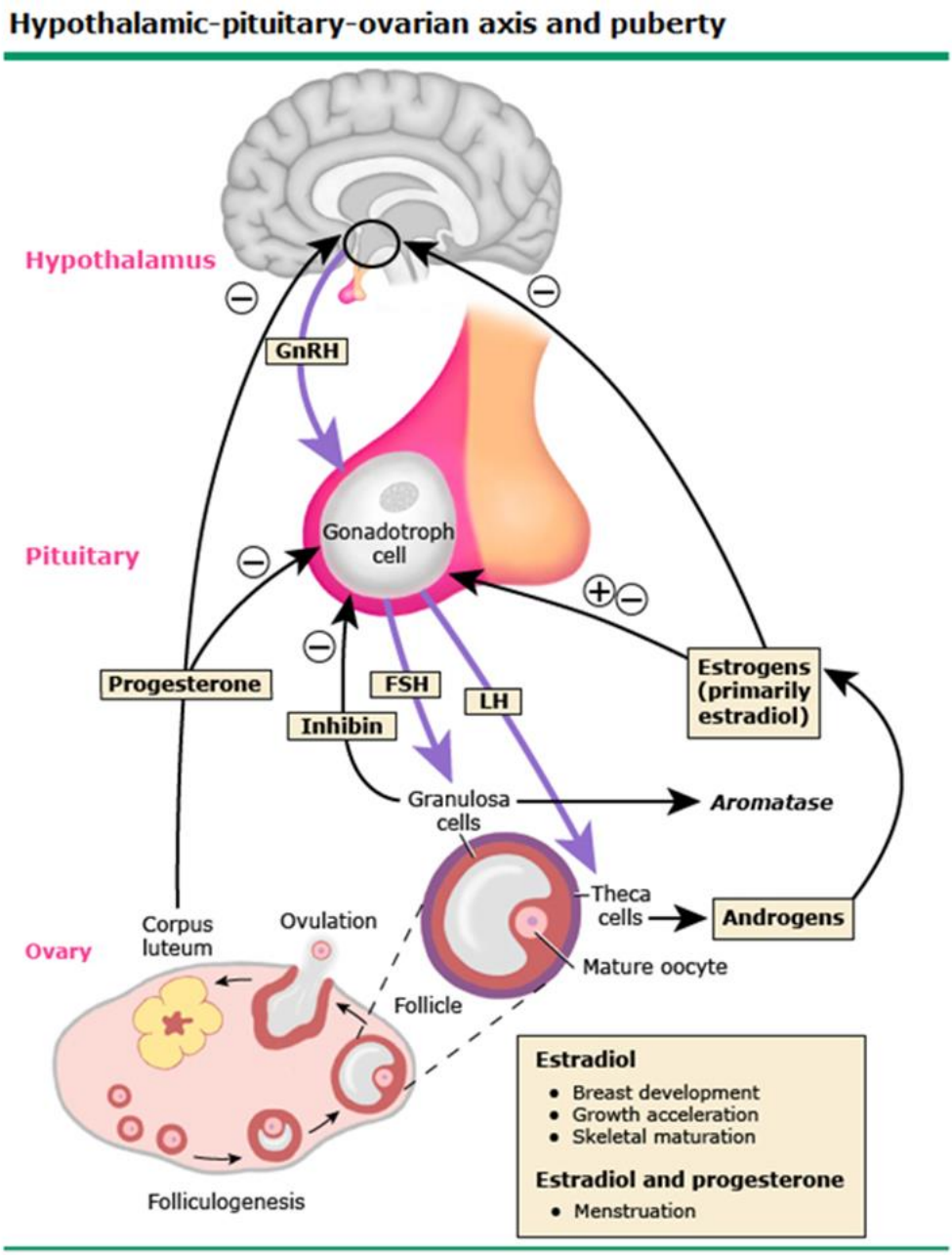


Figure 1-11. Normal Puberty in Females. (From Up to Date (107)). GnRH pulses from the hypothalamus increase in frequency, triggering release of FSH and LH from the anterior pituitary. FSH acts to mature the ovaries and increases the release of oestradiol, along with LH. Oestrogens act to increase breast development and growth. There is an eventual maturation and co-ordination of this system resulting in ovulation and cyclical menstruation. GnRH-Gonadotrophin releasing hormone; FSH-follicle stimulating hormone; LH-Luteinising hormone.

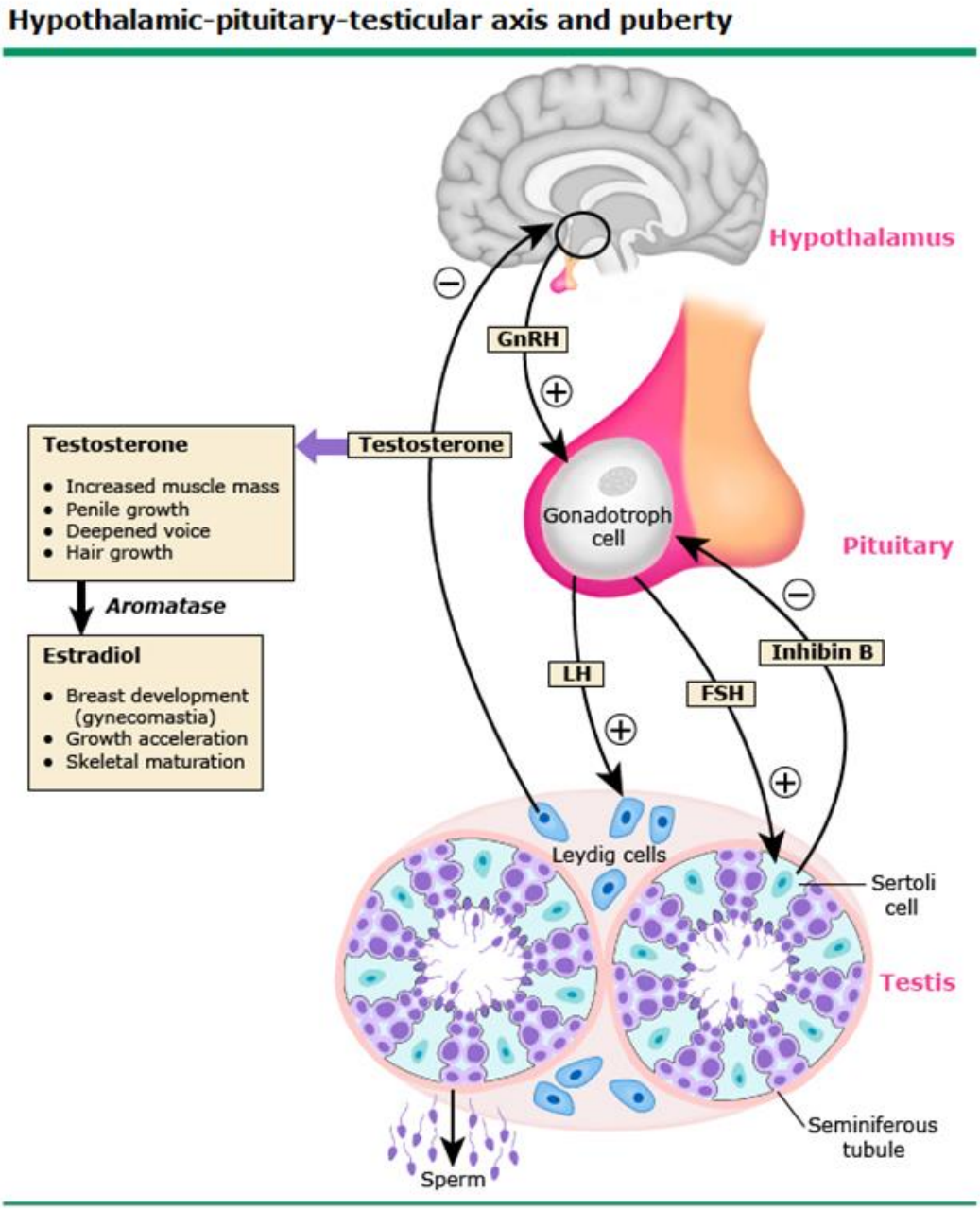


Figure 1-12. Normal puberty in boys (From Up to Date (107)). A cyclical increase in GnRH triggers the release of FSH and LH, which mature the testes and stimulate the production of testosterone. GnRH-gonadotrophin releasing hormone; FSH-follicle stimulating hormone; LH-luteinising hormone

1.1.5.2. Mini puberty of infancy

In young infants, there is a transient activation of the hypothalamic, gonadal pituitary axis in what is termed a 'mini-puberty' (Fig. 1-12). There is a rise in gonadotrophins after birth which peaks at 3 months of age(108). There is a subsequent transient rise in the levels of testosterone in boys and oestradiol in girls (109). Levels rise remarkably high, similar to those seen in puberty, and can even result in vaginal bleeding in some female infants, and virilisation

in some male infants(110). Levels of sex hormone and gonadotrophin thereafter drop to barely detectable levels throughout childhood, until the onset of puberty.

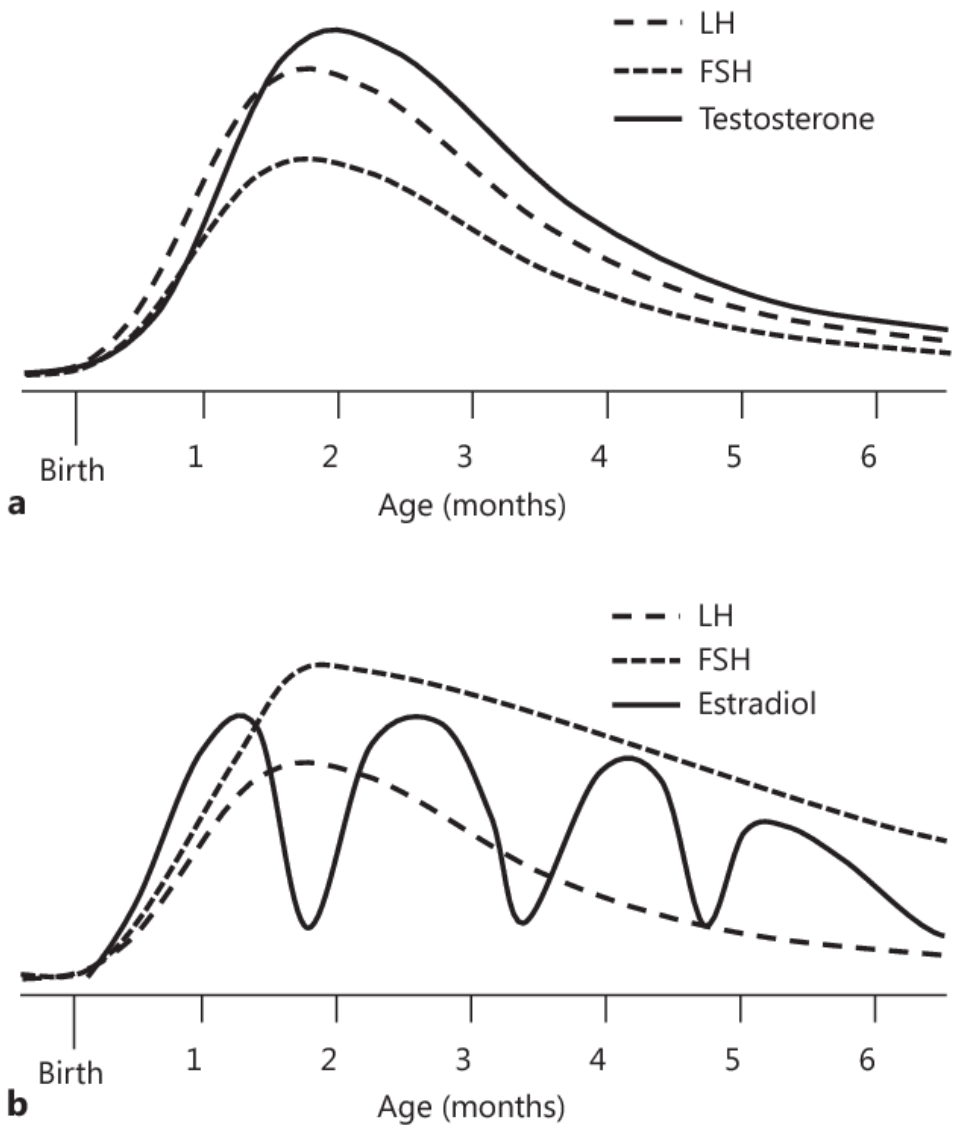


Figure 1-13. The mini-puberty of infancy (From Kuiri-Hänninen et al (110)). There is a mini-puberty in early infancy that associates with increased levels of gonadotrophins and sex steroids in a. male and b. female infants. FSH-follicle stimulating hormone; LH-luteinising hormone

1.1.5.3. Puberty and the immune system

Very little is known about the development of the immune system specifically over puberty. We know that from studying the immune system in children, adults and the elderly that changes occur with age and development. New-born babies have immature innate and adaptive immune responses which mature over childhood, to ultimately produce an adult phenotype. In the elderly, there is an immune senescence that occurs, with weakening of many adaptive and innate responses(111).

Despite puberty being a time of massive physiological change, there is almost no data about whether the immune system changes over these few years. This is probably associated with the lack of involvement of adolescents in research, as well as the disconnect between child and adult focussed care. This gap in the literature is a lost opportunity, as puberty provides a great model for studying the effects of increasing concentrations of sex hormones in males and females. Another group of young people who may help us better understand the effects of sex hormones and sex chromosomes, are young people with gender dysphoria.

1.1.5.4. Gender dysphoria

Gender dysphoria refers to the significant distress experienced by a person with a discordance between their assigned and experienced gender (112, 113). Young people with gender dysphoria often identify as being 'transgender' i.e. trans female/trans girl /trans woman in those born phenotypically male and trans male/trans boy/trans man in those born phenotypically female.

The diagnostic and statistical manual of mental disorders version 5(DSMV) , defines gender dysphoria as a discordance of an individuals assigned and experienced gender, along with significant distress or impairment in everyday functioning for at least 6 months, along with the presence of at least 6 of the criteria listed in Table 1-9 below(114).

Criteria for the diagnosis of gender dysphoria in children and adolescents
-A strong desire to be of the other gender or an insistence that one is the other gender.
-A strong preference for wearing clothes typical of the opposite gender.
-A strong preference for cross-gender roles in make-believe play or fantasy play.
-A strong preference for the toys, games or activities stereotypically used or engaged in by the other gender.
-A strong preference for playmates of the other gender.
-A strong rejection of toys, games and activities typical of one's assigned gender.
-A strong desire for the physical sex characteristics that match one's experienced gender.
-A strong dislike of one's sexual anatomy.

Table 1-9. Criteria for the diagnosis of gender dysphoria in children and adolescents (From DSMV(114)). The diagnosis of gender dysphoria requires at least 6 of the above criteria, with a significant impairment in functioning or distress due to gender dysphoria.

Referrals of young people to specialist gender dysphoria clinics are becoming much more frequent in the UK(115), probably due to an increased awareness and acceptance of these young people in society.

After thorough multidisciplinary assessment, upon reaching Tanner stage 2 of pubertal development, young people with gender dysphoria are offered pubertal suppression therapy with GnRH agonists(115). This therapy delays their pubertal development as their birth sex and prevents the development of irreversible secondary sexual characteristics. It works well as a potentially reversible tactic to prevent the development of unwanted characteristics (e.g. breast development and voice deepening), while allowing more time for full psychosocial evaluation and support prior to deciding about further, irreversible gender transition. It is shown that young people with gender dysphoria at presentation have significantly poorer psychosocial functioning than gender typical or cisgender young people, but that this improves with psychosocial support and even more so with pubertal suppression therapy(116).

After at least a year on puberty blocking therapy, and after extensive psychosocial input and support, young people with the capacity to consent may be offered cross-sex hormone therapy (oestrogen in those born phenotypically male and testosterone in those born phenotypically female)(115). The cross-sex hormone therapy is introduced slowly in line with standard pubertal induction regimens in endocrinology and allows for the induction of trans secondary sexual characteristics as well as the pubertal growth spurt. Trans females are usually kept on a GnRH agonist during this time, while oestrogen is introduced, as testosterone may not be sufficiently suppressed, whereas GnRH agonists may be withdrawn from trans males. Young patients are usually referred on to adult services, where further decisions can be made regarding full gender affirming surgery.

The long term biological outcome of transgender people who have received cross-sex therapy to induce trans puberty are poorly understood. Of note, there are 2 case reports of the development of SLE in trans females receiving oestrogen therapy (117, 118). This however is not enough to consider that oestradiol may be a real risk to trans females to develop SLE, and probably is in line with background population risk.

These young people provide an ideal model to investigate the relative contributions of X chromosome number and sex hormone environment to a range of biologic variables that show sex and pubertal differences. There has been a real interest in studying these young people in the fields of metabolic medicine and immunology (119). Just as immune deficiency syndromes have helped to demonstrate the inner workings of the immune system, transgender young people may provide a model for us to better understand the relative contributions of sex hormone and X chromosome number to immune functioning and disease manifestation.

1.1.5.5. Summary

There are massive physiological and hormonal changes that occur over puberty, but there is almost no data exploring whether the immune system changes over puberty. This is a lost opportunity, in the quest to explore the effects of sex hormones on the immune system. In addition, young transgender people may provide a good model of the variation of puberty, uncoupling the traditional link between X chromosome number and sex hormone environment.

1.1.6. Sex, puberty, hormone, X chromosome and the TLR7 mediated production of type 1 IFN.

In the preceding sections, the concepts of the immune system, sex, puberty, X chromosomes and hormones have been outlined. The known changes in the adaptive and innate immune system with sex or puberty, and highlighted the difficulty examining the underlying causes for this have been described.

The following section will focus specifically on the type 1 IFN, antiviral, innate immune system, and explore what is known about how sex, puberty, hormones and X chromosomes affect type 1 IFN production. As shall be discussed later, type 1 IFN is important in the development of SLE, and it can be argued that sex or pubertal differences in type 1 IFN production could help explain the increased prevalence of jSLE in females at puberty.

1.1.6.1. TLR7 mediated IFNα production is higher in females

It is well established that adult females produce more type 1 IFN after TLR7 signalling when compared to males(16). In the first and largest paper to show this in 2006, Berghofer and colleagues demonstrated that in 120 adult healthy volunteers, PBMC from females produced more IFNα than males, specifically after TLR7 and not TLR9 signalling. Resiquimod (R848) was used as a synthetic TLR7 agonist and CpGODN2216 as a synthetic TLR9 agonist. R848 is also an agonist of TLR8, although pDC, the chief producers of type 1 IFN do not express TLR8. To confirm that this effect was TLR7 specific, in the same paper, the experiment was repeated in a separate population of 101 healthy volunteers, using a different TLR7 (and non-TLR8) agonist, imiquimod (R837), which confirmed that females produced more IFNα than men after TLR7 stimulation specifically. They went on to investigate whether these differences were due to differences in the numbers of pDC between men and women but found no difference, and further showed that purified pDC still produced more IFNα in women than men. In addition, they tested for the production of TNFα after TLR7 stimulation, which showed no significant differences. They went on to further examine the role of sex hormone and *TLR7* gene expression in this difference, which I will demonstrate in subsequent sections.

This study was the first of its kind to describe this TLR7 specific sex difference in IFNα production. The volunteers assessed were from a wide age range (18-65) which include post-menopausal volunteers. They did not assess for any differences with age or menopause in this population to assess if females still produced more IFNα than men after menopause, to establish whether this difference only existed during reproductive maturity, which would imply a link with sex hormones. In addition, these ligands, although highly specific, were synthetic, and may not represent real life viral scenarios.

Following on from this, in 2009, Meier and colleagues investigated whether sex differences in TLR7 activation by HIV derived ligands might be responsible for the observed sex difference in HIV disease(120). For TLR ligands, they used an inactive HIV vesicle control and the HIV derived TLR7 agonists, $Gag_{RNA1166}$ and $Gp160_{RNA2093}$, or TLR9 agonist, CpGODN2219, to stimulate PBMC and measured pDC specific IFNα production by flow cytometry in 20 female and 23 male adults. They showed that a higher percentage of pDC from females produced IFNα after TLR7 stimulation, which was not true for TLR9 stimulation or TLR7 induced TNFα production(120). This confirmed the findings of Berghofer et al in that females produced more IFNα (and not TNFα) after stimulation with a real-life pathogen-derived TLR7 agonist, but not a TLR9 agonist. This finding was further confirmed by Seillet and colleagues in 2012 in 20 female and 15 male healthy adults(121).

All of these studies usually used antibodies towards IFNα2, making it unclear if this sex effect was subclass specific. Using a different synthetic TLR7 agonist, CLO97, Ziegler et al confirmed that there was a significantly higher pDC expression of all 13 IFNα subtypes and IFNβ in adult females, compared to males(122) implying that this effect was true for all known type 1 IFN.

All of these studies only involved adult participants, further highlighting the need to assess for these differences outside of sexual maturity, in order to establish if these differences are inherent to the female sex or associated with sex hormones. The only exception to this was a study in 2012 by Wang et al(123). They used PBMC from 2 month old infants and similarly showed that, after R848 stimulation, there was an increased percentage of pDC producing IFNα in female when compared to male infants(124). As discussed earlier however, 2-month-old infants do not provide a good model if one is trying to exclude the hormonal differences between the sexes, as they are going through the 'mini-puberty' stage and have high levels of sex steroid hormone.

The data therefore strongly indicates that females produce more IFNα after TLR7 stimulation than males. It is however unclear if there is an inherent different between the sexes, or

whether this only manifests during sexual maturity, which would a priori imply a hormonal basis for this difference.

1.1.6.2. Oestradiol and TLR7 induced IFNα production

After it was shown that females produced more IFNα in response to TLR7 stimulation than men, Berghofer et al went on to investigate whether varying doses of oestradiol in vitro affected the production of IFNα(16). They found that oestradiol and the oestradiol antagonist, ICI182780, had no effect on the ability of PBMC to produce IFNα after TLR7 stimulation(16). This was an important step in that it implied that the sex difference existed regardless of exposure of PBMC to oestradiol during stimulation but did not exclude the constitutive oestradiol environment that the cells had originated from.

In the paper by Meier et al, after it was shown that females produced more IFNα after TLR7 stimulation, they also reported a non-significant trend toward lower percentages of IFNα producing pDCs in response to TLR7 ligands in postmenopausal women compared to premenopausal women (mean 16.9% versus 27.6%, p = 0.08)(120). It is difficult to comment on this as the effect is not statistically significant, but it does imply a trend towards the effect of oestradiol *in vivo*.

This led Siellet et al to investigate the effect of oestradiol on TLR7 induced IFNα production in a mouse model(121). They found that in sub-lethally irradiated NOD/SCID/β2m$^{-/-}$ mice transplanted with female human progenitor cells, female donor human derived pDC produced more IFNα in recipient female mice compared to recipient male mice. They concluded from this that TLR7 induced pDC IFNα production was oestradiol related. Although this may be true, the assumption is flawed in that they only used female human donors, and therefore did not fully rule out the effects of X chromosome difference. In addition, they showed that, in 17 post-menopausal females, treatment with oestradiol for 3 months increased the percentage of pDC producing IFNα after TLR7 signalling. This effect was not specific though, as they also showed that there was an increase in pDC TNFα production after TLR7 stimulation, as well as an increase in the TLR9 induced IFNα production, which does not mirror the original TLR7 specific sex effect. Although this study showed that oestradiol may play a role in the sex difference seen in TLR7 induced IFNα production, it was not specific to IFNα nor TLR7 and did not explore the effect of the X chromosomal origin of the pDC.

The same group extended these data and in 2014 published a follow up paper(68). Human neonatal cord blood from both sexes was transplanted into the same irradiated NOD/SCID/β2m$^{-/-}$ recipient mice. After stimulating bone marrow cells with TLR7 agonist R848, regardless of the sex of the recipient mouse, pDC from female cord blood donors showed an

increased percentage of pDC producing IFNα (and not TNFα). When male recipient mice with male recipient pDC received oestradiol, there was a trend towards an increase in the percentage of pDC producing IFNα, but this was not significant. These data are important as they are the first to try and tease out the separate effects of X chromosome number and sex hormone, and show that regardless of hormone, donor pDC with two X chromosomes produced more IFNα after TLR7 stimulation. This is important, but it is a highly artificial model, with potential confounding factors. Mouse experiments are important in immunology, but do not however always reflect human physiology (125).

Therefore, there is evidence that implies that oestradiol may play a role in the increased TLR7 mediated production of IFNα, although this is not conclusive. In addition, there is an intriguing possibility that X chromosome number may inherently associate with TLR7 induced IFNα production. This highlights the need to examine whether young pre-pubertal children, with low hormone levels, show the same sex difference in TLR7 mediated IFNα production, and whether this changes over puberty. In addition, young transgender people and those with TUS may contribute to this model, by providing a human model which uncouples X chromosome number and serum sex hormone concentration.

1.1.6.3. Testosterone and TLR7 induced IFNα production

As is usually the case in the data regarding the hormonal differences in the immune system, there is fewer data regarding testosterone and its role in the sex difference in TLR7 induced IFNα production.

The only study to explore testosterone as a potential contributor to the sex difference seen in TLR7 induced IFNα production was the study by Wang et al, which assessed infants(124). After initially showing that TLR7 mediated IFNα production was lower in pDC from male infants, compared to females, they postulated that perhaps the 'mini-puberty' associated with infancy may contribute to this sex difference, specifically due to higher levels of testosterone in male infants. They tested this *in vitro* by pre-stimulating human female pDC with 3 doses of dihydrotestosterone (DHT), the active tissue metabolite of testosterone for 1 hour before stimulating with R848. Remarkably, they reported a negative correlation between IFNα production as measured by enzyme linked immunosorbence assay (ELISA) and DHT dose. This correlation with DHT was not seen for the production of TNFα after R848 stimulation but was reported for CpG (TLR9) stimulation.

This in vitro finding was intriguing, as it was not seen when oestradiol was added to the experimental system by Berghofer et al(16) and implies that testosterone may associate with a decrease in the TLR7 induced IFNα production in females. This further highlights the need to

explore the role of oestradiol, testosterone and X chromosome number independently in humans, *in vivo*.

1.1.6.4. TLR7 is coded on the X chromosome.

Equally intriguing is the fact that *TLR7* is coded for on the X chromosome. It stands to reason that perhaps differences in expression of *TLR7* gene between males and females could account for the increased TLR7 associated IFNα production seen in females. Given the data presented above, whereby the X chromosome, or portions thereof could escape X inactivation in some immune cells, this stands as a logical possibility.

With relatively small sample numbers, both Berghofer(16) (n=16) and Laffont(68) (n=10) could not demonstrate a sex difference in *TLR7* gene expression in pDC from healthy adults when analysing expression by PCR. Berghofer et al, using an EBV immortalised B cell line from one single female donor, showed that *TLR7* did not escape X inactivation in immortalised B cells or pDC in that donor(16). As discussed, the X chromosome mosaicism may not be in a steady state depending on the tissue type and individual, implying that a single donor and a single cell line may not be adequate to exclude the bi-allelic expression of *TLR7* in females.

In contrast to this, Zang et al showed that *TLR7* did escape X inactivation in immortalised B cells from large datasets containing many individuals(126), highlighting probable inter-individual heterogeneity and the need for larger sample sizes.

Interestingly, Souyris et al have recently shown that *TLR7* escapes bi-allelic expression in up to 30% of pDC, B cells and monocytes from healthy adult women(127). In addition, they have demonstrated that males had a lower PBMC protein expression of *TLR7* compared to women, when analysed by western blot analysis.

These data are intriguing and imply that *TLR7* gene expression may be bi-allelic in different cell types, clonal populations, tissues, and even show inter and intra- individual variability. It implies that X chromosome bi-allelism may be more dynamic than previously thought, especially in the X linked immune genes. This raises the question about whether the expression of X linked immune genes, specifically *TLR7*, may change with puberty and its associated hormonal changes, or display a steady expression regardless of sexual maturity.

1.1.6.5. Summary

It has been robustly shown that immune cells from adult females produce more type 1 IFN after TLR7 stimulation than males. The data suggests that this may be due to X chromosome number as well as sex hormone concentration, but this has not been definitively demonstrated in humans. There is a need to investigate whether this sex difference exists prior to puberty,

and whether it changes over puberty and to specifically assess for the contributions of sex hormone number, serum testosterone and oestradiol concentrations. This is particularly pertinent in the research of jSLE, a disease that is more common in females, manifests over puberty and displays a type 1 IFN transcriptional signature. As will be discussed later, jSLE is mediated and perhaps initiated by the type 1 IFN pathway and it therefore stands to reason that sex and pubertal differences in this pathway could be linked to the sex and pubertal bias seen in jSLE. A clinical overview of jSLE will be provided below in 1.3 before focussing on the emerging data that indicate that jSLE may be IFN mediated in 1.4.

1.3. Juvenile systemic lupus erythematosus.

Juvenile systemic lupus erythematosus (jSLE) is the prototypical, multisystem autoimmune disease characterised by auto-antibodies to nuclear self-antigens. It has been traditionally defined as an autoimmune disease- i.e. predominantly involving the adaptive immune system, but as data emerges regarding the importance of the innate immune system in jSLE, it can be argued that it has a more autoinflammatory origin, as will be discussed further(1). jSLE is defined as SLE with onset before the age of 16, and accounts for approximately 15% of the incidence of all SLE(128). jSLE has a more severe disease phenotype and disproportionately affects women and ethnic minorities, and most commonly presents around puberty. A brief overview of the epidemiology, clinical features and treatment strategies in jSLE is given below.

1.3.1. Epidemiology of jSLE

The incidence and prevalence of jSLE varies around the world from 0.3-0.9/100000, although the global data are scarce(4). Regardless of geographical location, the epidemiology of jSLE shows a variation with sex, age and ethnicity. In fact, it can be argued that jSLE targets groups that are traditionally under-represented in clinical and pre-clinical research, that is, adolescents, females and non-white ethnic groups.

1.3.1.1. Sex

SLE is more common in females at all ages, but the ratio between males and females varies with age. In 2016, Ambrose et al compared 924 individuals with SLE (413 jSLE, 511 adult-onset SLE (aSLE) (Fig. 1-14)(4). They report a sex ratio (female: male) of 5:1 in children (<12), increasing to 7:1 during adolescence (12-17) and settling at adult ratios of approximately 13:1 which is widely reported in adult data.

In the UK jSLE cohort, there are currently upwards of 400 patients enrolled with jSLE. In a publication in 2012, the characteristics of 276 of these were described, with a female to male ratio of 5.6:1 after 10 years of age and 2.7:1 before 10 years (129). The increase in the female

bias after sexual maturity does imply that sex hormones may play a role in the susceptibility to jSLE.

Supporting this observation, in mouse models of SLE, female mice develop SLE more quickly, have a more severe disease phenotype and die sooner(130). If these mice undergo ovarectomy pre-puberty, the disease is not as severe and develops later. However, if mice undergo ovarectomy post-puberty, the disease phenotype is unchanged, implying that puberty itself may influence the development of jSLE. In some mouse models, treatment with oestrogen antagonism improves disease, whereas oestrogen therapy worsens disease(131).

Interestingly, men with Klinefelter syndrome (XXY) have been reported to have a similar prevalence of aSLE as females (approximately 14 times higher than XY men) (132). Furthermore, 7 of 286 men with aSLE were confirmed to have Klinefelter syndrome, which is almost ten times higher than the general male population prevalence(133). In relation to the discussion above, this implies that having two X chromosomes might also be associated with the development of jSLE.

Characteristic	***All JSLE***	*Childhood*	*Adolescent*	***All adult***	*Adult*	*Mature*
Numbers	**413**	136	277	**511**	467	44
Female:Male	**6:1**	5:1	7:1	**13:1**	13:1	11:1
Median age	**13**	10	14	**31**	30	54

Figure 1-18. The sex ratio of SLE varies with age (From Ambrose et al(4))

1.3.1.2. Ethnicity

There is a well described ethnic variation in the incidence and prevalence of aSLE. In a review of adult jSLE epidemiology studies in the USA, the incidence in white people ranged from 3.3-5.6 (per 100000 cases), while the prevalence ranged from 43-56 (per 100000 cases)(134). In contrast, incidence African Americans ranged from 8-16, whereas prevalence ranged from 111-261. In 2016, Rees et al published a retrospective cohort study in the UK, based on 7732 cases of aSLE(135).They showed incidence and prevalence in white people at 6.73, and 134 respectively. In contrast, people of black, Afro-Caribbean origin had an incidence and prevalence of 31 and 517 respectively (135). Despite good data from western populations that aSLE affects non-white people more frequently, there are almost no good epidemiological data from the developing world and Africa(136).

Studies have persistently shown that non-white aSLE patients are more at risk of developing lupus nephritis, neurological lupus, and have a higher mortality(137).

The data from children and young people is similar to adults, in that jSLE seems to be more common in non-white population groups. In fact, in the LUMINA (LUpus in MInorities: NAture versus nurture) study in the US, there was a significantly higher proportion of African American patients with jSLE compared to aSLE. It has also been reported in the UK that aSLE patients were significantly more likely to be white than jSLE patients (4).The data from the UK jSLE cohort study from 2012 is represented in Table 1-10 below, and shows that white children had the lowest standardised incidence of jSLE, despite being the most represented in the group(138). In addition, it has been shown that non-white patients with jSLE display a worse phenotype, with a higher rate of renal and neurological disease, as well as an increased risk of having a catastrophic event as a presenting feature (139, 140). Despite this, there is a relative under-representation of non-white people in the jSLE research literature.

Ethnic group	No. (%) of patients	Standardized incidence per 100,000 persons per UK ethnic population
Caucasian	103 (52)	0.1
Chinese	4 (2)	0.8
Bangladeshi	5 (2.5)	0.9
Black Caribbean	8 (4)	0.7
Indian	18 (9)	0.9
Black “other”	2 (1)	1
Pakistani	12 (6)	0.8
Mixed race	15 (8)	1.1
Black African	19 (10)	2
Asian “other”	12 (6)	2.5
Not stated	0 (0)	–

Table 1-10. Ethnicity of patients in the UK jSLE cohort study(138)

1.3.1.3. Age

jSLE accounts for approximately 15% of all SLE(128). There are differences reported in disease severity and outcome in jSLE compared to aSLE. In a Korean study of 201 patients, fever, oral ulcers, nephritis, anaemia, and thrombocytopenia were more common in jSLE patients than aSLE(141). In a Spanish cohort of 3,428 aSLE patients and 484 jSLE patients, jSLE had significantly more renal and neurological involvement than aSLE. jSLE patients more frequently underwent all SLE-related treatment and procedures, as well as dialysis and kidney transplantations(142).In the LUMINA study mentioned above, jSLE was more active than aSLE at all timepoints, despite the fact that the young people with jSLE had lower self-reported disease activity than their adult counterparts. In addition, the LUMINA cohort showed an

increased rate of neurological and renal involvement as well as neurological and renal damage in jSLE as compared to aSLE(128). Most disturbingly, the jSLE group had double the mortality rate of the aSLE group (128). In the large UK cohort report by Ambrose et al, jSLE had higher rates of renal and neurological involvement, as well as almost six times the standardised mortality rate of aSLE (Figs. 1-15/16(4)).

The median age of onset of jSLE in the UK jSLE cohort group was 12.8 years of age in females and 11.1 years of age in males(138). In females, this closely approximates to the median age of onset of puberty mentioned above.

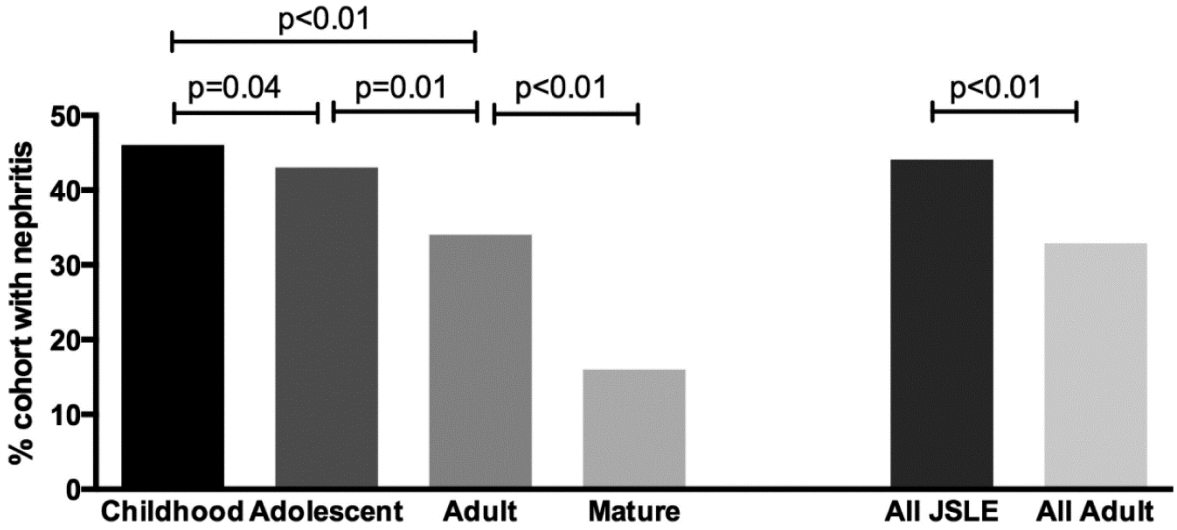


Figure 1-15. jSLE has an increased rate of renal involvement than aSLE (From Ambrose et al(4))

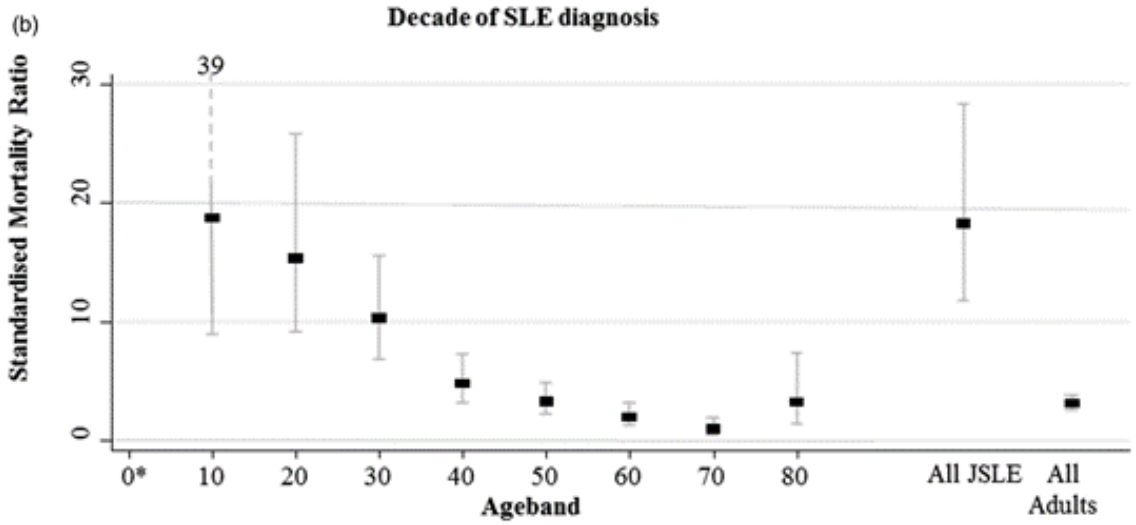


Figure 1-16. jSLE has a higher reported standardised mortality rate than aSLE (From Ambrose et al(4))

1.3.2. The clinical manifestations of jSLE

jSLE is a markedly heterogeneous multisystem disease that is unpredictable in its clinical phenotype. Nevertheless, there are certain unifying characteristics which allow for the classification of jSLE.

1.3.2.1. Classification Criteria

Although there are no validated diagnostic criteria for SLE, classification criteria exist, which were developed for adult onset SLE. The initial criteria for SLE were the America College of Rheumatology (ACR) criteria updated in 1997(143). New Systemic lupus international collaborating clinics classification (SLICC) criteria were developed in 2012(144). These are summarised below in Table 1-11. These criteria were not developed for jSLE, but they have been tested in young people, in a multicentre study where the sensitivity and specificity of the ACR criteria were 76.6% and 93.4%, respectively, whereas those of the SLICC criteria were 98.7% and 85.3%, respectively (145).

ACR criteria(143)		**SLICC criteria(144)**	
4 out of 11 required for diagnosis of SLE		4 out of 17 required, including at least one clinical criterion and one immunological criterion, OR biopsy proven lupus nephritis	
Criterion	**Definition**	**Criterion**	**Definition**
		Clinical criteria	
Malar rash	Fixed erythema, flat or raised over malar eminences, tending to spare nasal folds	Acute cutaneous lupus	Malar rash, bullous lupus, photosensitive rash, TEN, maculopapular lupus rash OR subacute cutaneous lupus
Photosensitivity	Skin rash as result of unusual reaction to sunlight		
Discoid rash	Erythematous raised patches with adherent keratotic scaling and plugging, atrophic scarring may occur	Chronic cutaneous lupus	Discoid, hypertrophic lupus, lupus panniculitis, mucosal lupus, chilblain lupus
		Non-scarring alopecia	Diffuse thinning or hair fragility with visible broken hairs in the absence of another cause
Oral ulcers	Painless oral or nasopharyngeal ulcers	Oral or nasal ulcers	Palate, buccal, tongue or nasal ulcers in the absence of another cause
Arthritis	Non-erosive arthritis involving two or more joints characterised by tenderness, swelling or effusion	Joint disease	Synovitis (swelling)or tenderness and morning stiffness in two or more joints
Serositis	Pleuritis or pericarditis	Serositis	Pleuritis or pericarditis
Renal disorder	Proteinuria (>3+ or >500mg/24hrs) or cellular casts (Red cell, haemoglobin, granular, tubular or mixed)	Renal	Proteinuria (Urine protein: creatinine ratio or 24 hr urine >500mg/24hr) or red blood cell casts
Neurological disorder	Seizures or psychosis without associated drug or metabolic cause	Neurological	Seizures, psychosis, mononeuritis multiplex, myelitis, neuropathy or acute confusional state in the absence of other causes
Haematological disorder	Haemolytic anaemia or leukopenia, or lymphopenia or thrombocytopenia	Haemolytic anaemia	
		Leukopenia or lymphopenia	
		Thrombocytopenia	
		Immunological criteria	
ANA positive		ANA positive	
Immunological disorder	Presence of the following antibodies; Anti-DsDNA or Anti-Smith or antiphospholipid antibodies	Anti-DsDNA	
		Anti-Smith	
		Antiphospholipid antibodies	
		Low complement	Low C3, low C4 or low CH50
		Direct Coombs test positive	

Table 1-11. ACR vs SLICC criteria for SLE (adapted from up to Date(146))

A new classification system is under development which attempts to improve the specificity and sensitivity of the current systems. This system will be weighted, with each criteria given a score, whereas the current criteria are equally represented currently(147).This new classification may include constitutive symptoms such as fever, and acknowledge that anti-nuclear antobody (ANA) positivity may be required for the diagnosis of SLE.

In any event, the classification criteria give an overview of the most common clinical features of the disease and highlights its multisystemic nature. Because virtually any organ may be involved in SLE, and its heterogenous nature, it has been given the moniker, 'the great imitator'(148). As mentioned above, young people are more likely to present with renal disease and neurological disease, are more likely to have disease associated damage and increased mortality(128).

1.3.2.2. Measures of disease activity in jSLE

There are multiple measurements of disease activity that have been developed in SLE. These include the SLE disease activity index (SLEDAI)(149), the British Isles lupus assessment group index (BILAG)(150)and the systemic lupus activity measure (SLAM)(151). All 3 have been shown to be sensitive to change and are sensitive measures of disease activity in children (152). SLEDAI is the simplest to use, as it is a simple additive score, and is often used for research purposes and clinical monitoring. It consists of 24 outcome measures, including 16 clinical measures and 8 laboratory measures. The organ measures are weighted differently, for example, neurological involvement is scored at 8, kidney involvement at 4 and skin involvement at 1. SLEDAI is scored according to the 30 days prior to scoring.

Clinical biomarkers such as the titre of anti-double stranded DNA antibodies (anti-DsDNA) and the complement split products C3 and C4 are routinely used in clinical practise as monitoring tools for disease activity, but a review of the literature has failed to show their consistency, or ability to predict flares(153). They are however often used by clinicians in association with other scoring systems such as the SLEDAI to monitor a patient's disease.

Composite scores exist which are routinely used in clinical trials. These include the SLE response index (SRI)-4 (154), the SRI-50(155) and the BILAG-Based Composite Lupus Assessment (BICLA)(156).

1.3.3. Treatment of jSLE

1.3.3.1. General

Young people with jSLE require specialist multidisciplinary care, appropriate to their developmental stage, and with a careful focus on their psychosocial and family context. jSLE is

an incurable disease that will have a long-term consequences, either due to disease or therapy, and young people need a supportive and professional multi-disciplinary team to manage them safely through childhood, adolescence and the transition into adult care. A detailed discussion about specific therapies is beyond the scope of this introduction, but the main themes along with successes and failures of some of the clinical trials which may give insights into the pathogenesis of jSLE will be highlighted here.

In general, supportive measures are advised, which includes vaccination and avoidance of UV exposure, which may trigger disease flares.

1.3.3.1.1. Anti-malarials

The most consistently effective drug for SLE currently is hydroxychloroquine (HCQ), a drug repurposed from its use as anti-malarial in the Second World War. While more targeted therapies have recently failed clinical trials, HCQ has consistently been shown to be an effective agent to decrease flares, morbidity and other complications in SLE(157).

It has been shown that anti-malarials are capable of binding nucleic acid in the endosome, thereby masking the TLR binding ligand(158). HCQ has been shown to have many other immune modulatory effects, including interfering with cell signalling (159) and lysosomal acidification, which interrupts proteolysis, chemotaxis, phagocytosis, antigen presentation and macrophage mediated cytokine production (160-162).

1.3.3.1.2. Glucocorticoids

Cortisol was the first glucocorticoid isolated from the adrenal gland in the 1940s, and the discoverers Kendall, Richstein and Hench were awarded the Nobel prize for this discovery in 1950(163). Glucocorticoids were rapidly shown to be effective in rheumatoid arthritis and asthma, and thereafter shown to be potent in the treatment of SLE(164). They have widespread immune suppressive properties, but these come at the price of a poor side effect profile. Side effects include hypertension, diabetes, weight gain, striae and Cushingoid features, hyperlipidaemia, glaucoma, osteoporosis, myopathy and avascular necrosis. Steroid withdrawal may precipitate a life threatening adrenal crisis. In addition, in children specifically, steroid therapy interferes with growth, pubertal development, bone density and ultimate adult height. This is in addition to the psychological distress a Cushingoid appearance can cause an adolescent. Glucocorticoids are so intrinsically linked to adverse events in SLE that it is difficult to tell how much damage is disease induced vs steroid induced. In the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI) measurement tool, half of the 12 domains included in the index relate at least in part to the damage induced by steroid therapy (165).

Glucocorticoids are however lifesaving in SLE and there is no doubt that they have contributed to the decrease in mortality over the decades. There is however a need to be mindful of their danger when treating a young person, and an urgent need for novel ideas to develop equally potent but less toxic drugs for use in SLE.

1.3.3.1.3. Immunomodulatory drugs in SLE

Cyclophosphamide, mycophenylate and azathioprine are non-specific immune suppressive drugs that are used in SLE(166). They may be used to induce disease remission or maintain remission, typically for severe organ involvement, i.e. nephritis or neurological disease and are recommended in multicentre jSLE consensus guidelines (167, 168). They do however also have their own set of side effects, including lymphopenia, infertility and teratogenicity, all of which are difficult to manage in young people.

1.3.3.1.4. Biologic agents in SLE

As more data has emerged about the immune system in SLE, there has been an increase in the number of targeted monoclonal antibody therapies that have been investigated for SLE (and jSLE). Despite promising outcomes reported anecdotally by physicians, these agents have largely not succeeded in clinical trials. In fairness, these drugs have been held up to much higher levels of scrutiny than corticosteroids or HCQ were when they were first discovered. This has led to a situation where only one new agent, belimumab, has been approved for the use of SLE in the last 60 years.

Despite this, clinically important findings have been detected in sub- and post-hoc analyses from these “failed” randomised control trials. This has highlighted the limitations in SLE trial design, including heterogeneity of disease, inadequate trial size or duration, insufficient dose finding prior to enrolment, choice of primary endpoints, under-estimation of placebo effect and non-standardized use of background therapy, including corticosteroid dose(169). In an excellent review on the subject. Mahieu et al have summarized the major biological therapy randomised control trials in Table 1-12(169).

Of note, recently sifalimumab(170), an IFNα monoclonal antibody and anifrolumab(171), an IFNAR antibody were successful in phase II studies, reaching their primary end points. In addition, there was a differential response, based on whether patients displayed an upregulated IFN gene signature or not. Despite these data, the company has released an early press release reporting that the first arm (multi-dose) has failed to reach primary endpoints after 1 year(172). It will still be interesting to see whether there was any differential response to these therapies based on stratification by IFN signature when the full results are released.

Table 1-12 Summary of drug trials in lupus adapted from Mahieu et al (169)				
Drug	**Target**	**Mechanism**	**Study**	**Outcome**
Rituximab	CD20	Deplete CD20+ B-cells	Merrill et al.(173)(EXPLORER) Phase II/III RCT Moderate-to-severe SLE 1° endpoints: a) Major or b) partial clinical response at 52 weeks	1° endpoints not met Pre-specified subgroup analysis found better response in blacks and Hispanic group treated with rituximab vs placebo Strict definitions for major and partial clinical response Amount of background therapy mitigated differences between treatment arms
Abatacept	CD80/86	Inhibit T-cell co-stimulation	Merrill et al.(174) Phase IIb RCT in mild-to-moderate SLE 1° endpoint: ≥1 BILAG A or B flare during 52 weeks	1° endpoint not met; similar flare rates with abatacept and placebo Improvements in PROs (health-related quality of life, fatigue, and sleep) with abatacept
Atacicept	BAFF/BLyS and APRIL	Neutralize BAFF/BLyS (TNFSF13B) and APRIL (TNFSF13A)	Isenberg et al. (APRIL-SLE) (175) Phase II RCT Moderate-to-severe SLE 1° endpoint: ≥1 BILAG A or B flare during 52 weeks	1° endpoint not met Atacicept 75mg and placebo had similar flare rate Atacicept 150mg arm halted for adverse events, but signal of fewer flares relative to placebo
Belimumab	BAFF/BLyS	Neutralize BAFF/BLyS (TNFSF13B)	Wallace et al.(176) Phase II RCT Active SLE 1° endpoints: a) Change in SELENA-SLEDAI score from baseline to 24 weeks and b) time to first flare (SFI) in 52 weeks	1° endpoints not met; guided choice of patient selection and endpoints for phase III RCTs Serologically active patients (ANA or anti-dsDNA antibody positive) had significant improvement in SELENA-SLEDAI and 2° endpoints at week 52 Led to development of SRI composite endpoint Unlimited changes in immunosuppressants and GC confounded results 1° endpoint met; greater SRI-4 response with belimumab vs placebo in both studies
			Navarra et al (177). and Furie et al.(178) (BLISS-52 and BLISS-76) Phase III RCTs Moderate-to-severe seropositive SLE 1° endpoint: SRI-4 response at 52 weeks	First use of SRI composite endpoint Stricter control of GC and immunosuppressants near end of study Pre-specified analyses showed improvements in HRQOL, fatigue29 Pooled post-hoc analysis suggested benefit in renal disease54
Tabalumab	BAFF/BLyS	Neutralize BAFF/BLyS (TNFSF13B)	Isenberg et al.(179) and Merrill et al.(180)(ILLUMINATE 1 and 2) Phase III RCTs Moderate-to-severe SLE 1° endpoint: SRI-5 response at 52 weeks	ILLUMINATE 1 did not meet 1° endpoint ILLUMINATE 2 met 1° endpoint; greater SRI-5 response with tabalumab vs placebo Neither study met key secondary endpoints (fatigue, time to flare, GC dose) Patients with any change in immunosuppression dose, including decrease, were considered non-responders Development suspended
Epratuzumab	CD22	Alter B-cell responsiveness	Wallace et al. (181)(ALLEVIATE 1 and 2; SL0006 open label extension) Phase II RCTs Moderate-to-severe SLE 1° endpoint (modified): BILAG response with no treatment failure at week 12	Enrolment suspended early for low supply of study drug; 1° endpoint evaluated at 12 weeks instead of intended 24 weeks Greater achievement of 1° endpoint with epratuzumab than placebo Improvements in HRQOL and reduced GC doses at 48 weeks34

			Wallace et al.(182) (EMBLEM) Phase IIb dose-ranging study Moderate-to-severe SLE 1° endpoint: BICLA response at 12 weeks	First use of BICLA composite endpoint Not powered for significance, but suggested efficacy and safety of 2400mg combined monthly dose More homogeneous patient population than prior RCTs
			Clowse et al(183). (EMBODY 1 and 2) Phase III RCT Moderate-to-severe SLE 1° endpoint: BICLA response at 48 weeks	1° endpoint not met; no difference in BICLA response rate and secondary efficacy measures between epratuzumab and placebo
PF-04236921	IL-6	Neutralize IL-6	Wallace et al.(184) Phase II RCT Moderate-to-severe SLE 1° endpoint: SRI-4 at 24 weeks	1° endpoint met for 10mg dose; also showed improvements in BICLA and HRQOL (secondary pre-specified endpoints) Reduction of flares (SFI) with 50mg dose 200mg treatment arm discontinued for serious adverse events
Edratide	Unknown	Unknown	Urowitz et al.(185) Phase II RCT Mild-to-moderate SLE 1° endpoint: Reduction in SLEDAI-2K and Adjusted Mean SLEDAI (AMS) through 26 weeks	1° endpoint not met, but significant improvements in BILAG were seen (secondary pre-specified endpoint) Background GC use may have confounded results
Sifalimumab	IFNα	Neutralize some species of IFN-α	Khamashta et al.(170) Phase IIb RCT Moderate-to-severe SLE 1° endpoint: SRI-4 response at 52 weeks Statistical significance set at p<0.098	1° endpoint met; greater achievement of SRI-4 and improvements in skin disease, joint count, and fatigue with sifalimumab compared to placebo
Anifrolumab	Type 1 IFN receptor	Neutralize type 1 IFN activity	Furie et al.(186) Phase II RCT Moderate-to-severe SLE 1° endpoint: SRI-4 response at 24 weeks and sustained reduction in GC dose	1° endpoint met; more SRI-4 responders and reduced GC doses with anifrolumab than placebo Effect size greater in patients with high IFN at baseline
			Phase III RCT underway (TULIP)	Recent press release –TULIP1 Arm- failed to reach statistically significant decrease in SRI4 at 12 months(172)
Ustekinumab	IL12/23	Neutralises IL12/23	Van Vollenhoven et al (187) Phase IIRCT 1° endpoint SRI4 at	1° endpoint achieved. No requirement to reduce GC dose. Not all patients on standard therapy, response above standard therapy unclear.
Baricitinib	Jak1 and 2 inhibitor	Neutralises jak1 and 2 and prevent STAT phosphorylation	Wallace et al(188) Phase II RCT, 2mg and 4mg groups 1° endpoint resolution rash/arthritis at week 24	4mg-1° endpoint met along with reduction in SRI4 at 24 weeks, less flares. GC dose high in both groups, tapering not used as an endpoint. No stratification.

1.3.4. Summary

jSLE is an unpredictable, severe, multisystem disease, that targets females, ethnic minorities and is more common around the onset of puberty. Although treatments exist, they are not universally effective and have severe side effects. There is an urgent need for novel ideas and therapies in the treatment of jSLE. The sex bias in jSLE, as well as the increased onset around the age of puberty may provide new insights into disease pathogenesis and potentially inform target development.

1.4. Type 1 IFN in the pathogenesis of jSLE

There is no doubt that SLE is highly heterogeneous and that multiple abnormalities in multiple immune compartments contribute to the pathogenesis. There is emerging data that the innate, antiviral immune system, specifically the type 1 IFN pathway may contribute to disease development in jSLE, with some arguing that it may be the underlying immune-pathogenetic mechanism(1). Given the above sex difference described in the TLR7 mediated type 1 IFN pathway, one could hypothesise that this may be responsible for the known sex difference described in jSLE. As it is unknown whether the type 1 IFN pathway changes with puberty, it is unknown whether this may associate with the pubertal association seen in jSLE.

In working towards developing a hypothesis, the known genetic, transcriptomic and epigenomic data in the SLE literature will be summarised, with a focus on how the type 1 IFN pathway emerges as an important pathway in the pathogenesis of jSLE. Known abnormalities in the adaptive immune system in SLE will then be briefly mentioned, before concentrating in more detail on the type 1 IFN pathway.

With the exception of the important work done by the Pascual group(189), the majority of the research regarding type 1 IFN has been performed in aSLE. Therefore, the pathogenesis of SLE will be referred to in general, and it shall be specified if a study included patients with jSLE.

1.4.2. The genetics of SLE

SLE has a 24% concordance in monozygotic twins, vs a 2% concordance in dizygotic twins(190). Genes in the MHC region confer the strongest risk, followed by non-MHC genes. Non MHC genes include those involved in immune complex processing, DNA degradation, apoptosis, clearance of cellular debris, neutrophil and monocyte signalling, TLR/type 1 IFN signalling, nuclear factor kappa beta activation, B and T cell function and signalling(191)(some examples Fig. 1-17(192)). Importantly, more than half of the non-MHC loci are associated directly with the IFN pathway, or indirectly in terms of increased availability of DNA/RNA/nuclear cell debris

and apoptotic waste. The number of described polymorphisms continues to increase as more studies are done. It has been estimated, in a recent large Taiwanese population study (23 million participants and 18283 SLE cases), that heritability accounted for 43% of the phenotypic variance of SLE (193). Currently the GWAS data accounts for approximately 28% of the heritability of jSLE, leaving almost a third unaccounted for. This could be explained by smaller effect variants, rarer variants not present in common arrays, and epigenetic modifications(191). All genome wide association studies (GWAS) in SLE have been in adult patients, and consist of multiple large scale studies in European and Asian ethnicities, highlighting the need to include children and African patients in these studies (191).

GWAS-identified SLE susceptibility genes

Pathway(s)	Loci implicated in SLE and other autoimmune diseases	Loci implicated only in SLE
Lymphocyte activation	*PTPN22*, *TNFSF4*, *IL10*, *SPRED2*, *STAT4*, *PXK*, *AFF1*, *IL12A*, *BANK1*, *TCF7*, *SKP1*, MHC genes, *IKZF1* and *IKZF3*, *BLK*, *ARID5B*, *CD44*, *LYN*, *ETS1*, *FLI1*, *SH2B3*, *CSK*, *ELF1*, *CIITA*, *ITGAM*, *TYK2*	*IKZF2*
IFN or Toll-like receptors	*IFIH1*, *PRDM1*, *UHRF1BP1*, *TNFAIP3*, *IRF5-TNPO3*, *IRF7* and *IRF8*, *SOCS1*, *PRKCB*, *UBE2L3*, *IRAK1*	None
Inflammation	*TNIP1*	None
Immune complex or waste clearance	*FCGR2A*, *FCGR2B*, *FCGR3B*, *ATG5*, *CLEC16A*	*NCF2*, *LYST*
Unknown	*ABHD6* (may be related to lymphocyte activation), *RAD51B* (may be related to IFN pathways), *MECP2* (may be related to IFN pathways), *RASGRP3*, *TMEM39A*, *PITG1*, *TNXB*, *JAZF1*, XKR6, *FAM167A–AS1*, *WDFY4*, unknown genes: rs1167796, rs463128, rs7186852, rs7197475	*SMG7* (may be related to interferon pathways), *DHCR7*, *NADSYN1*, *SLC15A4*, *PLD2*, *CXorf21*

GWAS, genome-wide association studies; IFN, interferon; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus.

Figure 1-17. GWAS-identified susceptibility genes classed into function pathways as per Tsokos et al 2016(192).

1.4.2.1. Rare monogenic lupus

There are much rarer monogenic forms of SLE which allow for valuable insights into pathogenesis, and equally point to the type 1 IFN pathway as an important in SLE pathogenesis. These have been broadly classed by Tsokos et al in a review and shown below(Fig.1-18(192)). Many of these include defects in cell debris or nucleic acid clearance through complement deficiencies, or abnormalities in the cell nucleic acid clearance mechanisms. Many of these defects cause an increased type 1 IFN gene signature, along with similar clinical manifestations to SLE.

Aicardi Goutierres syndrome(AGS) presents clinically with extremely early onset neurological disability with intracranial calcifications mimicking intra-uterine infection, fevers, skin ulcers, a type 1 IFN transcription signature and nuclear autoantibodies, similar to those seen in

SLE(194). It has been directly linked to defects in intracellular nucleic acid clearance through mutations in *TREX1, SAMDH, IFIH* and *RNAse-H2*(194). It has provided a direct link between the development of systemic inflammation, anti-nuclear antibodies, an abundance of endogenous nucleic acid substrate due to decreased clearance and a resulting increase in type 1 IFN production and ISG upregulation.

Similarly, familial chilblain lupus is a rare, autosomal dominant form of SLE with cold-induced inflammatory lesions presenting in early childhood. Familial chilblain lupus is usually caused by a mutation in TREX1 (3' repair exonuclease 1), similar to AGS and defective clearance of innate nucleic acids is implicated.

These familial defects in nucleic acid clearance that cause an SLE like phenotype with a type 1 IFN signature provide an important conceptual framework to the hypothesis that the type 1 IFN pathway may be the initiating pathway in the development of SLE.

Monogenic causes of SLE and lupus-like disease

Gene	Effect	Features	Pathway
C1QA, C1QB, C1QC	Complement C1 deficiency	Early-onset, severe SLE, infections; high penetrance*; AR	Immune complex and waste clearance
C1R, C1S	Complement C1 deficiency	Early-onset, severe SLE, infections; high penetrance; AR	Immune complex and waste clearance
C4A, C4B	Complement C4 deficiency	Early-onset, severe SLE, infections; high penetrance; AR	Immune complex and waste clearance
C2	Complement C2 deficiency	Infections, cutaneous disease; moderate penetrance; AR	Immune complex and waste clearance
C3	Complement C3 deficiency	Membranoproliferative glomerulonephritis; low penetrance; AR	Immune complex and waste clearance
CYBB	X-linked chronic granulomatous disease	Infections, chronic granulomatous disease; low penetrance; X-linked	Immune complex and waste clearance
PEPD	Xaa-Pro dipeptidase deficiency	Cutaneous ulcers; low penetrance; AR	Immune complex and waste clearance
MAN2B1	Lysosomal α-D-mannosidase (laman) deficiency	Hearing loss, dysostosis multiplex, progressive cognitive decline; low penetrance; AR	Lysosomal oligosaccharide catabolism
TREX1	Aicardi–Goutières syndrome 1	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR or AD	Nucleic acid sensing; type I IFN
DNASE1	SLE	High penetrance; AD	Nucleic acid sensing
DNASE1L3	SLE 16	Early onset; high penetrance; AR	Nucleic acid sensing
SAMHD1	Aicardi–Goutières syndrome 5	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR	Nucleic acid sensing; type I IFN
ACP5	Spondyloenchondrodysplasia with immune dysregulation	Spondyloenchondrodysplasia, vitiligo, growth retardation; low penetrance; AR	Nucleic acid sensing; type I IFN
RNASEH2A, RNASEH2B, RNASEH2C	Aicardi–Goutières syndrome 4, 2, and 3 respectively	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR	Nucleic acid sensing; type I IFN
ADAR	Aicardi–Goutières syndrome 6	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AR or AD	Nucleic acid sensing; type I IFN
IFIH1	Aicardi–Goutières syndrome 7	Basal ganglia calcification, brain atrophy, skin ulcers, fevers; high penetrance; AD	Nucleic acid sensing; type I IFN
DDX58	Singleton–Merten syndrome 2	Dental loss, arterial calcification, joint contractures; high penetrance; AD	Nucleic acid sensing; type I IFN
TMEM173	STING-associated vasculopathy, infantile-onset	Skin ulcers, interstitial lung disease; low penetrance; AD	Nucleic acid sensing; type I IFN
ISG15	Immunodeficiency 38, with basal ganglia calcification	Mycobacteria, intracranial calcification; low penetrance; AR	Nucleic acid sensing; type I IFN
PSMB8	Nakajo syndrome	Fever, contractures, neutrophilic dermatitis; low penetrance; AR	Immune complex and waste clearance; type I IFN
FAS, FASLG	Autoimmune lymphoproliferative syndrome 1A and 1B, respectively	Autoimmune cytopenias, adenopathy; high penetrance; AD	Lymphocyte signalling
PRKCD	Autoimmune lymphoproliferative syndrome 3	Autoimmune cytopenias, adenopathy; moderate penetrance; AR	Lymphocyte signalling
PTPN11	Noonan syndrome 1	Short stature, cardiac anomalies; low penetrance; AD	Lymphocyte signalling
RAG1, RAG2	Several types of severe combined immune deficiency	Infections, granulomas; low penetrance; AR	Lymphocyte signalling

*Penetrance is indicted as a qualitative assessment of the percentage of people with the condition who have features of SLE. **AD, autosomal dominant;** AR autosomal recessive; IFN, interferon; SLE, systemic lupus erythematosus.

Figure 1-17. Summary of described monogenic lupus like diseases (From Tsokos et al (192)).

1.4.3. The epigenetics of SLE.

Analysis of the epigenetic processes in SLE have also highlighted the importance of type 1 IFN in the pathogenesis. Epigenetic processes govern how genes are expressed in a tissue and signal-specific manner(195)

Epigenetic processes include DNA methylation, histone modification and non-coding RNAs that regulate gene expression. These changes correlate with changes in chromatin accessibility and transcription factor binding(191).

In general, methylation in regulatory sequences induces gene silencing, while hypomethylation allows for transcriptional chromatin accessibility, and active gene expression when transcription factors are available(196). A genome wide methylation study of CD4 T cells in 36 adult volunteers (18 healthy, 18 SLE) identified significant hypomethylation in IFN-regulated genes in naïve CD4+ T cells from SLE patients, including *IFIT1, IFIT3, MX1, STAT1, IFI44L, USP18, TRIM22* and *BST2*(197). 21 out of 35 hypomethylated genes identified were regulated by type I IFN, and the hypomethylation of these was not related to disease activity (197). The same study included genome wide gene expression, which confirmed overexpression of these hypomethylated genes in total, but not naïve CD4+ T cells in SLE, suggesting epigenetic priming of IFN-regulated genes, which may lead to future hyper-responsiveness (197). A follow up study confirmed that IFN was the most significant unifying canonical pathway ($p = 6.41 \times 10^{-8}$), with IFNα as the most significant upstream regulator in these genes ($p = 6.77 \times 10^{-23}$)(198).

In addition, histone modification has been shown to be altered in SLE(199), and 635 of the sites with altered histone H4 hyperactivation, were potential binding sites for IRF1, a transcription factor downstream of type 1 IFN, once again linking the epigenetic modifications of SLE back to type 1 IFN.

1.4.3. The transcriptomics of SLE

Unlike, the epigenomic and genomic data, the original important transcriptomic description of an IFN signature in SLE by Bennett et al was in data from young people with jSLE (189). RNA transcription from 30 young people with jSLE and 9 healthy volunteers was analysed, and 15 gene transcripts were found to be significantly upregulated after stringent Bonferroni correction (Fig. 1-19). Among these, 14 were IFN inducible, or ISG, leading to the coining of the term, 'IFN signature' in jSLE. It was established in this relatively small cohort that this IFN signature correlated to disease activity, and was extinguished through high dose steroid therapy(189).

The second most commonly differentially expressed genes related to a granulocyte signature, which was surprising, as neutrophils are not classically included in the PBMC compartment.

Bennet et al then identified a group of smaller, low density granulocytes that co-purify with mononuclear cells in jSLE.

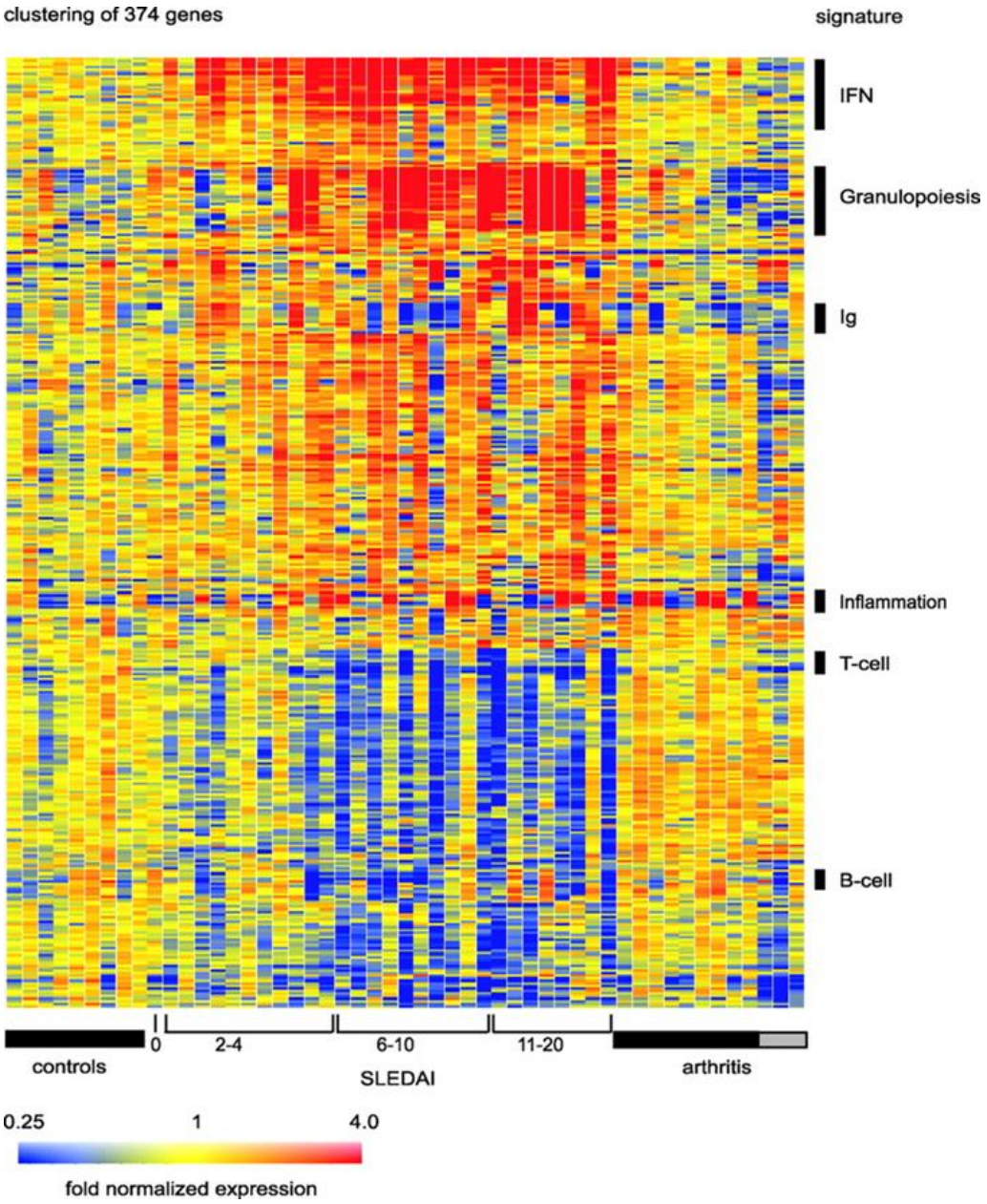


Figure 1-19: The IFN gene expression signature in jSLE as first described by Bennet et al(189).

The second important transcriptome paper in aSLE was published in the same year by Baechler et al (200). This confirmed the upregulation of expression of IFN inducible genes, as well as the correlation with disease activity. Importantly, in these data they assigned an IFN score to each volunteer and found that roughly half of the patients with aSLE displayed an elevated IFN score(200).

Kirou et al confirmed that the IFN induced genes overexpressed in aSLE are those that are predominantly induced by IFNα and not type 2 IFNs like IFNγ(201).

To complicate matters it has been show that the specific set of ISG upregulated in SLE varies widely in each immune cell subtype and in different ethnicities(202). There has been evidence that African American patients may be more likely to have an increased ISG upregulation, and a wider variation of ISG upregulated in SLE (202-204).

It has been shown that the upregulation of ISG is present in relatives of patients with jSLE, and shows heritability, which argues towards IFNα being a primary event in the pathogenesis of SLE(205)

It has been suggested that transcription profiles may be used to improve stratification of SLE as they are used in oncology to direct therapy(192). Recently, Banchereau et al longitudinally profiled the blood transcriptome of 158 jSLE patients (206). Using mixed models accounting for repeated measurements, demographics, treatment, disease activity, and nephritis class, they confirmed a prevalent IFN signature and identified an intermittent plasmablast signature as the most robust biomarker of disease activity. They reported gradual enrichment of neutrophil transcripts during progression to nephritis. In addition, they have described personalized immune-monitoring, which enabled patient stratification into seven groups, supported by patient genotypes. These are the first steps towards stratifying jSLE for trial design and eventual stratified therapy (206).

Therefore, transcription data from SLE show a predominant type 1 IFN signature, which is correlates with disease activity and may be useful in the stratification of SLE in the future.

1.4.3. The adaptive immune system in SLE.

Prior to the discovery of the IFN signature in SLE in the early 2000s, and due to the presence of auto-antibodies, it was widely held that SLE was caused by abnormalities in the adaptive immune system. Indeed, the type 1 IFN system is not the only deranged pathway in SLE. For the sake of completeness, some of the known abnormalities in the adaptive immune system will be briefly summarised here.

Multiple cytokines are deranged in SLE including IL-10, IL-18, B cell activating factor (BAFF), TNF, IL4, IL6 and others. The MHC locus is the one of the main genetic susceptibility foci for SLE. This underpins the importance of T cells in lupus pathogenesis. T cells subtypes, signalling pathways and activation are all known to be disrupted in SLE(192).

SLE is characterised by antibodies to self, specifically towards DNA, cell nuclei, and components of ribonucleoprotein particles(1). This has led to many investigations into the trigger of loss of self-tolerance in the adaptive immune system. The autoantibodies produced are class switched and of a high affinity, implying T cell help(192). These antibodies may be

present for years before the onset of disease and accumulate in the lead up to disease presentation (207). It is not only auto-antibodies that are high affinity in SLE, it has been shown that SLE patients produce higher affinity antibodies to influenza after vaccination, and may produce broadly neutralising antibodies to HIV (208, 209).

Indeed, targeting the adaptive immune system has led to the landmark U.S. Food and Drug Administration approval of belimumab(178), a drug that inhibits B lymphocyte stimulator, as well as anecdotal efficacy (despite failing clinical trials) of B cell depleting agents like rituximab or T cell activation inhibitors like abatacept(1).

Despite these important discoveries through the investigation of the adaptive immune system, auto-antibodies are not sufficient to produce disease in isolation. Targeting of the adaptive immune system has also only produced partial disease response.

Multiple investigations have pointed towards type 1 IFN as an early trigger for and crucial to the sustained abnormal immune response in SLE. I have shown above, that the anti-viral effects of type 1 IFN are ubiquitous within the cells of the immune system. Therefore, there have been recent theories that the persistent and excessive production of type 1 IFN and activation of molecular events downstream of the IFNAR are important to the early and sustained events in the adaptive immune pathways, and ultimately in lupus pathogenesis(1).

1.4.4. Type 1 IFN in SLE

Type 1 IFN has long been associated with the development of SLE. In 1969, Steinberg et al reported that in NZBW mice, with a predisposition to develop a lupus like phenotype, injection of polyinosinic·polycytidylic acid, a synthetic RNA and IFNα inducer, decreased the time to development of lupus features(210). In fact, in the abstract, they proposed a hypothesis, which years later we are again considering:

'According to this hypothesis, the unusual feature in this disease is not a unique virus, but rather the unique genetic susceptibility of the B/W (particularly female) host to immunization with nucleic acids. A similar pathogenetic mechanism may be operative in some humans with systemic lupus erythematosus'(210)

IFNα is maintained in serum at miniscule quantities and is not reliably detectable by available IFNα assays. For this reason, the direct amounts of *ex vivo* IFNα has not been reliably reported in serum from SLE patients. In 1979, Hooks et al ingeniously used the antiviral nature of IFNα to detect it in a vesicular stomatitis virus infection cell assay(211). They showed that IFNα was present at relatively higher titres in patients with SLE, and that it correlated with disease activity(211). Hua et al repeated this assay in 2006 and confirmed that there was also an

upregulation of ISG in the reporter cells, and blocking IFNα (but not IFNγ or IFNβ) abrogated this effect (212).

There have been multiple reports of cases of SLE induced by IFNα therapy (213-215). The published literature supports IFNα, as the most abundant type 1 IFN in patients with lupus(1). In addition, blocking IFNα (but not IFNγ) in SLE serum *in vitro* blocks the ability of the serum to upregulate ISG similar to blocking the IFNAR, suggesting that IFNα is responsible for the IFN signature in SLE(216). IFNβ and IFNγ are shown to be increased in SLE serum however, and their role in the disease has not been fully investigated(216).

In an important report in 2001 ,Blanco et al noted that monocytes behaved like dendritic cells (i.e. induced T cell proliferation) after exposure to serum from jSLE patients(35). They found that this effect was dependant on the presence of IFNα in the serum.

It is argued that in light of these findings, in addition to the genetic, epigenetic and transcription data provided above, that IFNα is essential in the pathogenesis of SLE, and may even act as the initiating factor. The next obvious question would be whether there is evidence of an increased expression of nucleic acid receptors in SLE, and whether, when stimulated, these produce an increased amount of IFNα. In addition, it would be important to investigate for evidence of increased sources of endogenous nucleic acid in SLE. The data pertaining to these questions is scarce, and mostly comes from animal models, but will be summarised below to highlight gaps in the literature.

1.4.5. The source of nucleic acids in SLE

There are various potential sources of nucleic acid ligand available for sensing and subsequent IFNα production in SLE, including apoptosis, increased endogenous retroelement (ERE) activity and increased release of neutrophil extracellular traps(NETs).

1.4.5.1. Apoptosis in SLE

SLE has been associated with defects in the clearance of apoptotic cell debris(217). The characterisation of monogenic complement deficient forms of SLE has also highlighted the importance of the clearance of nuclear cell debris in SLE. Apoptotic cell debris can trigger TLRs and other nucleic acid sensors, and apoptosis may be further influenced by infection, UV light exposure and other cytokines(192).

1.4.5.2. Endogenous nucleic acids

ERE comprise a large proportion of the human genome. Non-long terminal repeat (LTR) long interspersed nuclear elements (LINE) are the most abundant and represent almost a fifth of

the human genome (218). LINE are capable of autonomous transposition, and contain coding regions for RNA polymerase, RNA binding protein, reverse transcriptase and an endonuclease.

In the absence of virus, it has been proposed that ERE may act as an endogenous source of nucleic acid substrate in SLE(219). It has been shown that LINE RNA transfection is capable of inducing TLR7 mediated type 1 IFN production (220). In addition, the expression of ERE is enriched in patients with SLE and is related to disease activity, although it is unclear whether this is primary or associated with increased IFN, as LINE is IFN inducible(221).

1.4.5.3. Neutrophils

In SLE, neutrophils and low density granulocytes have been shown to readily produce NETS, whereby they extrude nuclear material as well as immune-stimulatory anti-microbial proteins(222).

1.4.6. Sensing nucleic acids in SLE

There is evidence emerging that *TLR7* expression and associated nucleic acid sensing is enhanced and may be pathogenic in SLE, whereas *TLR9* expression may be relatively protective. As we have shown, TLR7 specific IFNα production is increased in females, and *TLR7* is coded for on the X chromosome. Given the importance of IFNα in SLE, this naturally leads one to question whether TLR7 may be responsible for the sex bias in SLE.

1.4.6.1. TLR7 and TLR9 in SLE

It has been well described that self or foreign nucleic acids, bound to antibodies, are capable of entering the endosome and triggering IFNα production or ISG transcription via TLR7 or TLR9 (15, 223, 224).This effect has been shown in vitro to be dependent on FcγRIIa, diminished by RNA degradation and increased by NET anti-microbial proteins such as HMGB1 or LL37 (1, 225, 226).

When both *TLR7* and *TLR9* were inhibited in the (NZB × NZW) F1 lupus mouse model, development of disease was suppressed (227). A mutation in *Unc93b1*, which traffics TLR7 and TLR9 to endosomes also improved the lupus phenotype in C57VL/6(B6)-*Fas*^{lpr} mice (228).

In the important BXSB mouse model, male mice develop severe lupus-like autoimmunity (229, 230). It was shown that this depended on an area on the Y chromosome, named the Y-linked autoimmune accelerator (*yaa*) region which was found to contain a translocation of *TLR7* from the X chromosome onto the Y chromosome, resulting in a 2 fold increase in expression of *TLR7* in male mice (231-233). It was shown that this increased expression of TLR7 was responsible for the disease development.

Christensen et al showed that MRL/Mp$^{lpr/lpr}$ mice that lacked *TLR9*, did not develop anti chromatin antibodies, but developed more severe disease and early mortality (234). If the same mouse model lacked *TLR7* however, they did not develop anti RNA binding protein (RBP) antibodies, and all features of disease were improved (234). Santiago-Raber et al confirmed this with B6.Nba2 mice (235). If *TLR9* was absent, the mice developed a markedly accelerated form of lupus nephritis, associated with functionally upregulated expression of *TLR7*.Disease exacerbation in *TLR9* deficient mice was completely suppressed by the deletion of *TLR7*(235). Recently Celhar et al have confirmed that Sle1TLR9$^{-/-}$ mice develop severe disease, produce more class-switched antibodies and have a skewed autoantibody repertoire toward RNA-containing antigens(236). Sle1TLR9$^{-/-}$ mouse renal DCs were more efficient at TLR7 dependent antigen presentation and expressed higher levels of TLR7 protein. Importantly, this increase in *TLR7* gene expression occurred prior to disease development, indicating a role in the initiation stages of disease(236). This has led to the idea that expression of *TLR9* may be protective in lupus, whereas *TLR7* may be pathogenic. These data are from animal models, and this has been more difficult to show in humans.

In humans, the presence of auto-antibodies towards RNA associated proteins in humans (e.g. RNA binding protein, RBP), has been associated with a type 1 IFN signature in SLE (201), implying that RNA sensing (by TLR7 or others) is important in the production of IFNα and the upregulation of ISG.

Midgley at al showed that human PBMC *TLR9* and *TLR7* expression was increased in jSLE, but *TLR7* did not reach statistical significance(237). It is accepted that *TLR9* is an ISG, therefore one would expect it be increased in SLE, and the actual direction of the expression in SLE cannot be measured without correcting for ISG expression. Komatsuda et al also reported that *TLR7* and *TLR9* expression is increased in SLE, but once again, have not corrected for ISGs. In this paper, the expression of multiple genes has been tested, without correcting for multiple tests, making it unlikely that *TLR7* , with its small effect size (p=0.011) was significantly increased(238). Another large adult cohort reported the same findings, but once again did not control for the effect of ISG (239).

In 55 adult women and 40 adult men with aSLE, Kelley et al could show no difference in the gene copy number of *TLR7* when compared to matched healthy volunteers(240) .Other studies have shown no difference in *TLR7* copy number in jSLE(240) . A recent study of a large number of children in Mexico reported an increased risk of jSLE if the *TLR7* copy number variant was greater than 1 in males and greater than 2 in females, with an overall increase in *TLR7* copy number variant in jSLE (241).

Therefore, although the animal data indicate that *TLR7* gene expression is important in disease pathogenesis, the human data are less clear.

1.4.6.2. Cytoplasmic nucleic acid sensing in SLE

Information is emerging about the potential role of cytoplasmic nucleic acid sensors in SLE (242). Specifically, AGS has been associated with an abnormal sensing of nucleic acid in the cytoplasm. This may be due to increased availability of nucleic acids to cytoplasmic RNA or DNA sensors due to abnormalities in enzyme pathways responsible for nucleic acid degradation such as TREX1, RNaseH2, SAMDH and ADAR1(242). In addition, Aicardi Goutierres has been linked directly to a mutation in the MDA5 gene *IFIH(243).* This gives indirect insight into the potential for cytosolic nuclear sensing to be disrupted in SLE. These cytosolic sensors should therefore not be neglected when investigating the production of IFNα in SLE.

1.4.7. Nucleic acid induced type 1 IFN production in SLE.

Despite evidence of increased type 1 IFN in SLE, there is little consensus on the source of this IFN. In the original study by Blanco et al, when PBMC from jSLE patients were stimulated with influenza virus (ssRNA virus and TLR7 agonist), they produced the same amount of IFNα as healthy volunteers. In addition, depleting pDC from the system did not fully deplete the IFNα producing capacity as it had done in the healthy volunteers(35). It was also shown that herpes simplex virus (DsDNA virus and TLR9 agonist) induced pDC IFNα production was reduced in SLE(244).

There have been varying reports about whether TLR7 induced IFNα production is different in SLE (35, 244, 245). A recent large study involving 71 adult SLE patients and 45 healthy adults showed an increase in the TLR7 mediated pDC production of IFNα and TNFα in SLE(245). This study included patients with active disease in addition to those with low disease activity and reported that the TLR7 mediated pDC production of IFNα correlated with disease activity.

Therefore, there is evidence that IFNα is increased in SLE. *TLR7* gene expression associates with the development of SLE in animals, whereas the expression of *TLR9* is potentially protective. It is unclear whether the expression of *TLR7* is increased in humans with jSLE, and as *TLR9* is a known ISG, it is unclear whether it is truly increased after correcting for IFN signature. There is some evidence as to increased *TLR7* copy number conferring an increased risk to humans in developing SLE. Evidence is conflicting, but there is a possibility that TLR7 mediated IFNα production may be increased in SLE, and that this may correlate with disease activity.

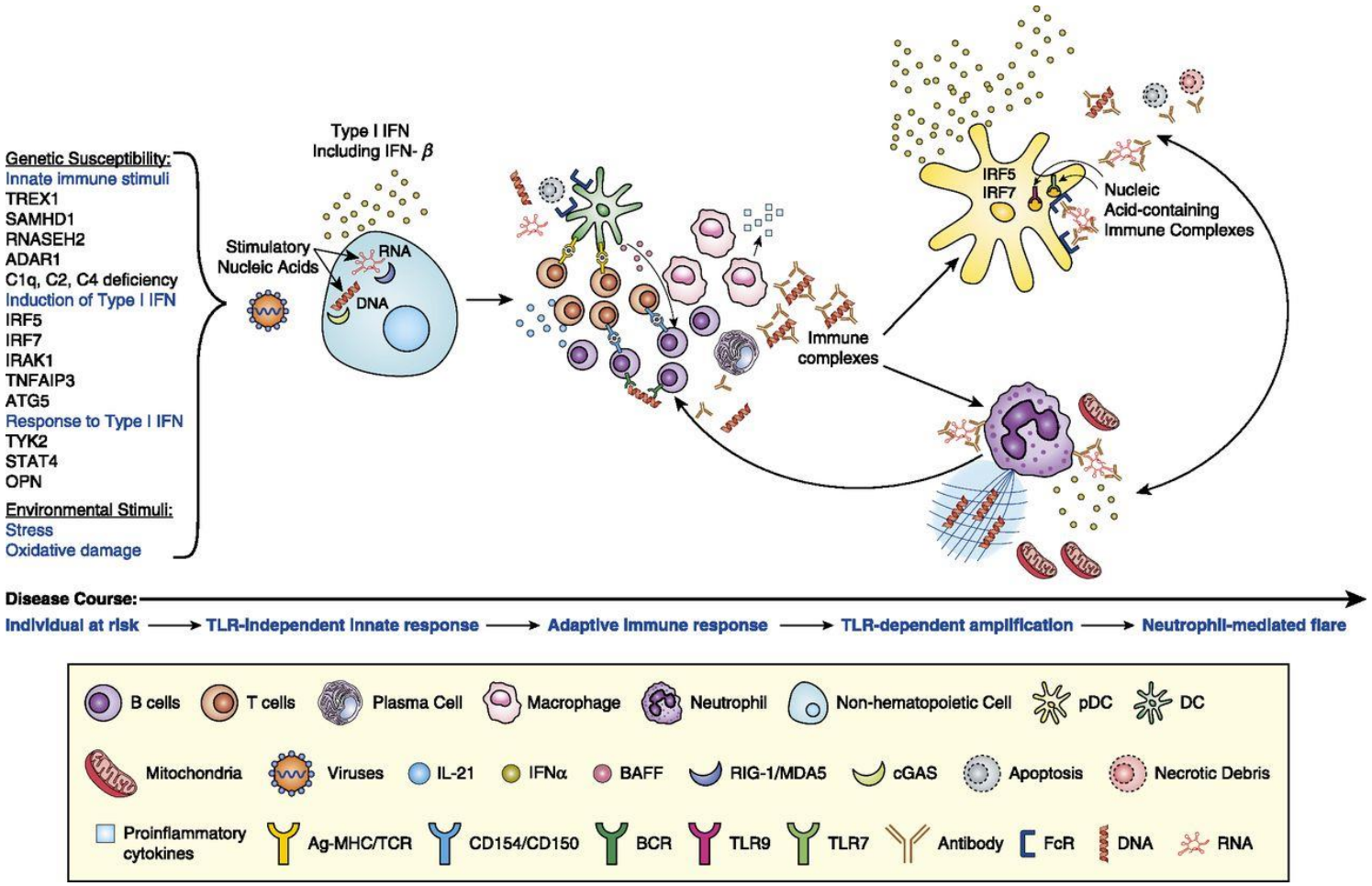
Therefore, there is a need to examine whether *TLR7* and *TLR9* gene expression and associated IFNα production is different in young people before puberty, which may provide insights into

the sex and pubertal bias in jSLE. In addition, there is a need to examine whether *TLR7* and *TLR9* gene expression and associated IFNα production is different between healthy young people and those with jSLE, in order to examine whether TLR7 expression and IFNα production may be pathogenic in SLE.

1.4.7. Type 1 IFN centric pathogenesis hypothesis

The data summarised above has led some researchers, to hypothesise an 'IFN centric' view of SLE, where numerous polymorphisms in the IFN pathway, associated with environmental triggers, set the stage for either endogenous or exogenous nucleic acids to trigger a type 1 IFN response(1). This then primes the adaptive immune system (which may have further risk polymorphisms in B or T cell function) to promote an anti-nuclear self-reactive environment. This leads to the presentation of auto-antibody bound self-nucleic acids through Fc dependant presentation to pDC which serves, along with the release of NETs, to further amplify the production of type 1 IFN (Fig. 1-20).

This emphasises the need to explore the effects of TLR7 mediated IFNα production in jSLE, and to assess whether these are responsible for the sex and pubertal bias seen in jSLE.



pDC-plasmacytoid dendritic cell, Il-interleukin, IFN-IFN, BAFF-B cell activating factor, RIG- retinoic acid-inducible gene, MDA5- Melanoma Differentiation-Associated protein 5, cGAS- cyclic GMP-AMP synthase, TLR-toll like receptor, Ag-antigen, MHC-major histocompatibility complex, BCR-B cell receptor

Figure 1-19. Type 1 IFN-centric view of SLE pathogenesis from Crow et al(1). Polymorphisms in nucleic acid degradation or type 1 IFN pathway genes, along with environmental stress stimuli enhance the activation of the type 1 IFN pathway. Endogenous nucleic acids activate cytoplasmic PRR and induce type 1 IFN which primes an adaptive immune response, in a host that may be enriched in gene variants that lower the threshold for lymphocyte activation. Resulting autoantibodies target nucleic acids or nucleic acid–binding proteins, immune complexes form and strongly amplify type 1 IFN production, predominantly IFNα, through FcR-dependent activation of pDCs mediated by endosomal TLRs. Neutrophils respond to type 1 IFN and immune complex exposure with extrusion of nucleic acids containing neutrophil extracellular traps. Neutrophils and pDCs each amplify production of type 1 IFN by the other cell type, modify the function of adaptive immune system cells, and amplify autoimmunity. The immunologic consequences of this sustained viral-like response include broad immune dysregulation,

1.5. Summary

Sex and puberty are neglected variables in immunology and data is lacking about their contribution to sex biased disease. In addition, the role of X chromosome number and sex hormone environment are difficult to separate out in human experiments.

It is clear that the type 1 IFN pathway is important in the pathogenesis of jSLE, a disease with a strong sex bias, which is more common during puberty. It is known, in adults, that females produce more IFNα after TLR7 stimulation. It is not known whether *TLR7* gene expression or TLR7 mediated IFNα production changes during puberty. There is a need to confirm the role of TLR9 and TLR7 in jSLE. There is also a need to investigate whether TLR7 gene expression or TLR7 mediated IFNα production changes over puberty, and to examine the relationship to X chromosome number and sex hormone, in a manner that may explain the sex and pubertal bias in jSLE.

Hypothesis

This thesis aims to test the following hypothesis:

Puberty associates with an increased TLR7 mediated type 1 IFN production in females.

Aims

1. To investigate whether sex and pubertal differences exist in:
- pDC activation and percentage
- pDC surface protein expression of known IFN inducible genes
- TLR7 or TLR9 induced pDC IFNα production
- pDC IFN pathway gene expression.
2. To investigate whether the above variables associate with X chromosome number or serum sex hormone concentration.
3. To investigate whether differences exist in the above variables between young people with jSLE compared to healthy young volunteers.

Chapter 2: Materials and Methods

2.1. Recruitment.

Young volunteers were recruited, and informed consent was sought with Research Ethics Committee approval REC11/0101. Healthy participants older than 12 years were recruited from the local community at science outreach events. Healthy participants less than 12 years old were only recruited if blood was being taken for an unrelated clinical indication (e.g. surgery for routine, non-inflammatory procedures i.e. dental extraction, circumcision). Exclusion criteria included acute or chronic illness and concurrent medication (including oral contraceptive).

Transgender young people on cross-sex hormone therapy (i.e. testosterone in those born phenotypically female and oestradiol in those born phenotypically male) and young women with TUS were recruited from endocrinology clinics. Exclusion criteria included acute or chronic illness.

Young people with low disease activity jSLE were recruited from rheumatology clinics. jSLE was defined as SLE presenting before the age of 16 and diagnosed by the treating clinician. Exclusion criteria included a prednisolone dose of >15mg/day and SLEDAI score > 5. SLEDAI was calculated for each individual. Briefly a clinical score was calculated based on clinical information in the 30 days preceding sample collection. This included a weighted score of 16 clinical indices and 8 laboratory indices.

Upon consent, demographic data, clinical data, and peripheral blood was collected. In addition, participants older than 13 completed a pubertal self-assessment questionnaire, based upon established pubertal developmental phases(100) (Fig 2-1/2-2). If participants were younger than 13, their parents either completed the form for the child, or with them if the parents thought it appropriate. In further analysis, volunteers were included who were pre-pubertal or post-pubertal according to self-classification.

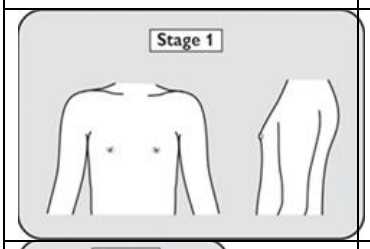
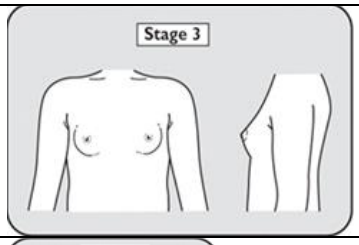
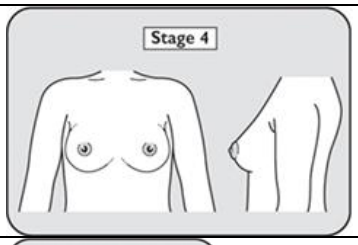
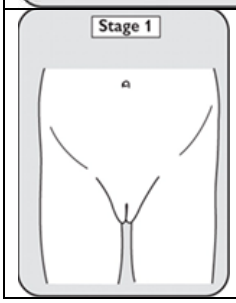
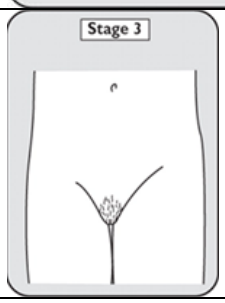
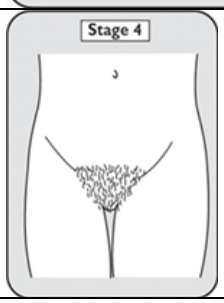
Blood was collected using blood collection vacutainers containing sodium heparin 102IU, (BD 367876) and silicon containing SSTII tubes containing gel (BD 367954). Not more than 2ml/kg of blood was taken in total from participants. Blood was processed at different times of day but all within 2 hours of collection from each volunteer.

Confidential self-assessment questionnaire

Female

Please indicate below which category best describes what stage of puberty you are at. This form is private and will have your study number, but not your name. It is important that you fill it out as truthfully as possible.

Please mark the category that best describes your stage of development in the boxes like this ☒

Pre-puberty (Tanner stage 1) *If **all** of the following*: ☐	**In Puberty** (Tanner stage 2-3) *If **any** of the following*: ☐	**Completing Puberty** (Tanner stage 4-5) *If **all** of the following:* ☐
-No signs of nipple or breast development -No pubic hair	-Any breast enlargement so long as nipples also enlarged -Any pubic or axillary (armpit) hair growth	Started periods(menarche) with breast, pubic and armpit hair development
		
		

Adapted from Butler, G, Kirk J. OSH Paediatric Endocrinology and Diabetes

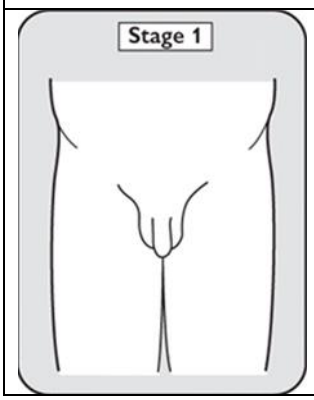
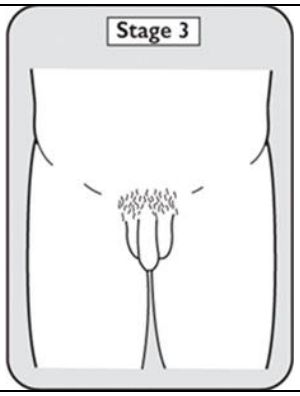
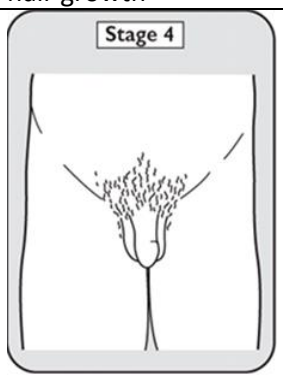
Figure 2-1. Female self-assessment questionnaire.

Confidential self-assessment questionnaire

Male

Please indicate below which category best describes what stage of puberty you are at. This form is private and will have your study number, but not your name. It is important that you fill it out as truthfully as possible.

Please mark the category that best describes your stage of development in the boxes like this ☒

Pre-puberty (Tanner stage 1) *If **all** of the following* ☐	**In Puberty** (Tanner stage 2-3) *If **any** of the following*: ☐	**Completing Puberty** (Tanner stage 4-5) ☐
-High voice -No growth of testes or penis -No pubic hair	-Slight voice deepening -Growth of the testicles -some growth of the penis -Beginnings of pubic or armpit hair growth	If **any** of the following: -Voice fully changed (broken) -Adult size of penis with pubic and armpit hair growth -Moustache and early facial hair growth
		

Adapted from Butler, G, Kirk J. OSH Paediatric Endocrinology and Diabetes

Figure 2-2. Male self-assessment questionnaire.

2.2. Human blood processing.

2.2.1. PBMC separation protocol.

Separation was carried out in a class 2 hood and sterile technique followed at all times. All reagents were made up sterile and only opened in the hood. All equipment was sterile, and all reagents were endotoxin-free.

Blood was diluted 1:1 with RPMI 1640 containing L-glutamine and $NaHCO_3$ (Sigma-Aldrich) supplemented 100 IU/µg/ml penicillin/streptomycin (Sigma-Aldrich).

Briefly, 15ml of Ficoll-paque plus (GE-healthcare GE17-1440-03) was added to a SepMate 50 tube (StemCell 85450), carefully pipetted through to fill the bottom of the tube. A maximum of 17ml of diluted blood was added slowly to the tube, on top of the Ficoll. The tube was then centrifuged at 1200g for 10 minutes at 20^{o}C with full acceleration and deceleration. The top layer was then quickly poured off into a new 50 ml centrifuge tube (Greiner T2318). Media was added to the tube to dilute up to 50ml in total. The tube was re-centrifuged at 1800RPM for 10 minutes at 20 ^{o}C at maximum acceleration and deceleration to wash. The cell pellet was re-suspended in 25ml of media containing RPMI 1640 containing L-glutamine and $NaHCO_3$ (Sigma-Aldrich) supplemented 100 IU/µg/ml with 10% heat inactivated fetal calf serum (FCS) (Biosera) and counted. Cells were then re-suspended at 10 million per ml in freezing media -10% dimethyl sulphoxide (Sigma 276855) and 90% heat inactivated FCS at -80° C in cryovials. Cryovials were labelled with the allocated sample number, date and cell volume and frozen in Nalgen 'Mr Frosty' freezing container (controlled freezing rate at 1°C per minute with isopropyl alcohol). The samples were stored in liquid nitrogen at 196 °C at no later than 72 hr post initial freezing.

2.2.2. Serum separation protocol

Whole blood was collected in vacutainers with silicon clotting agents that did not contain heparin (BD 367954). The blood was allowed to clot at room temperature for at least 30 min. Serum was transferred to sterile Eppendorf tubes (Sigma T2422) and micro-centrifuged at 10000g for 10 min at room temperature before labelling, freezing and storing at -80°C.

2.3. PBMC thawing and counting

PBMC were rapidly thawed in water at 37°C, washed once with RPMI and 10%FCS and re-suspended in media plus 10% FCS (RPMI 1640 containing L-glutamine and $NaHCO_3$ (Sigma-

Aldrich) supplemented 100 IU/µg/ml with 10% heat inactivated fetal calf serum (FCS) (Biosera)). Cells were counted with a haemocytometer.

2.4. Cell Culture.

PBMC were plated in a U bottomed 96 well plate at 1 million cells per well in RPMI 1640 containing L-glutamine and $NaHCO_3$ (Sigma-Aldrich) supplemented with 10% FCS (Biosera) and 100 IU/µg/ml penicillin/streptomycin (Sigma-Aldrich). One million cells were each stimulated with TLR7 agonist, R848 (Invivogen-tlrl-r848) at 1ug/ml or TLR9 agonist, CpGODN2216 (CpG) (Invivogen tlrl-2216) at 1µM or left unstimulated (in RPMI+10%FCS) for 20 hours. Brefeldin A (BFA) 5ug/ml (Sigma B7651) was added to the unstimulated and R848-stimulated cells at 0 hours. BFA was only added to the CpG stimulated cells at 16 hrs. Briefly, after 16 hours, the CpG stimulated cells were centrifuged (1800RPM for 3 min), media discarded and re-suspended in the new media (RPMI+FCS) containing BFA 5ug/ml (Sigma B7651).

2.4. Flow Cytometry.

2.4.1. Ex-vivo immune-phenotype flow cytometry.

Upon thawing, one million PBMC were plated in a 96 well plate. These were centrifuged at 1800 RPM for 3 min and re-suspended in phosphate buffered saline (PBS), and washed once. Thereafter cells were re-suspended in a 50ul mixture of live/dead fixable stain near infrared (423105) and the antibodies listed in Table 2-1 and incubated for 30 min in the dark at 4°C. Cells were washed once with PBS after incubation and re-suspended in 100ul FoxP3 Fix/perm solution (Ebioscience 421403) for 45 minutes in the dark at 4°C before one wash with permeability buffer 1:10 (Ebioscience 421403). A mixture of perm buffer and IRF 5 antibody (Alexafluor488; ab193245; clone-EPR6094; Abcam; 1.50) was added for 30 minutes in the dark before washing with perm buffer once and re-suspending in 100ul FACS buffer (PBS+0.1%FCS and 0.01%FCS and 1mM EDTA). Gating was set using isotype controls or full minus one samples. Gates illustrated in Fig 2-3 and 2-4. Tetherin is expressed constitutively on many cell types including pDC and B cells, and is upregulated with type 1 IFN stimulation(246). For this reason, tetherin median fluorescent intensity rather than percentage of cells positive, was measured and analysed (247)(Fig 2-5).

Antibody	Fluorochrome	Cat number	Clone	Company	Titre
Tetherin	APC	348410	RS38E	Biolegend	1:50
CD86	PeCy7	305408	IT2.2	Biolegend	1:50
CD4	BV711	317439	OKT4	Biolegend	1:100
BDCA2	PE	354203	201A	Biolegend	1:50
CD56	PerCP Cy5.5	318322	HCD56	Biolegend	1:100
CD11c	BV421	301627	3.9	Biolegend	1:100
CD8	V500	561618	SK1	BD	1:100
CD14	BUV 737	564444	M5E2	BD	1:100
CD19	BUV 395	563549	SJ25C1	BD	1:100
CD3	BUV 805	565515	UCHT1	BD	1:200

Table 2-1 Cell phenotype surface staining panel.

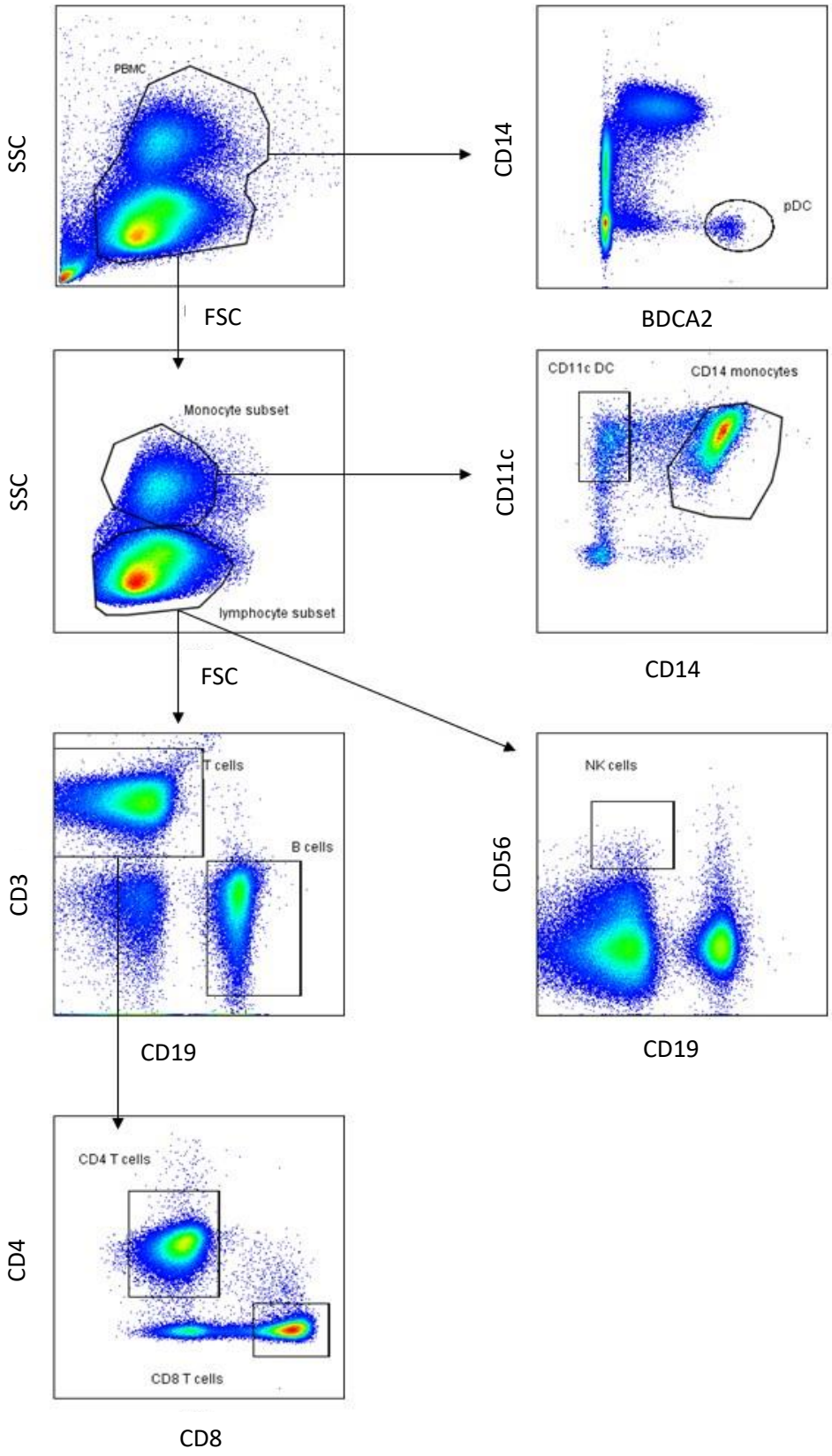


Figure 2-3. PBMC phenotype gating strategy *Ex vivo* PBMC were analysed by flow cytometry. Dead cells and single cells were excluded (not shown) before gating was performed as shown for BDCA2+ pDC, CD11c+ cDC, CD14+ monocytes, CD4+ T cells, CD8+ T cells, CD19+ B cells and CD56+ NK cells.

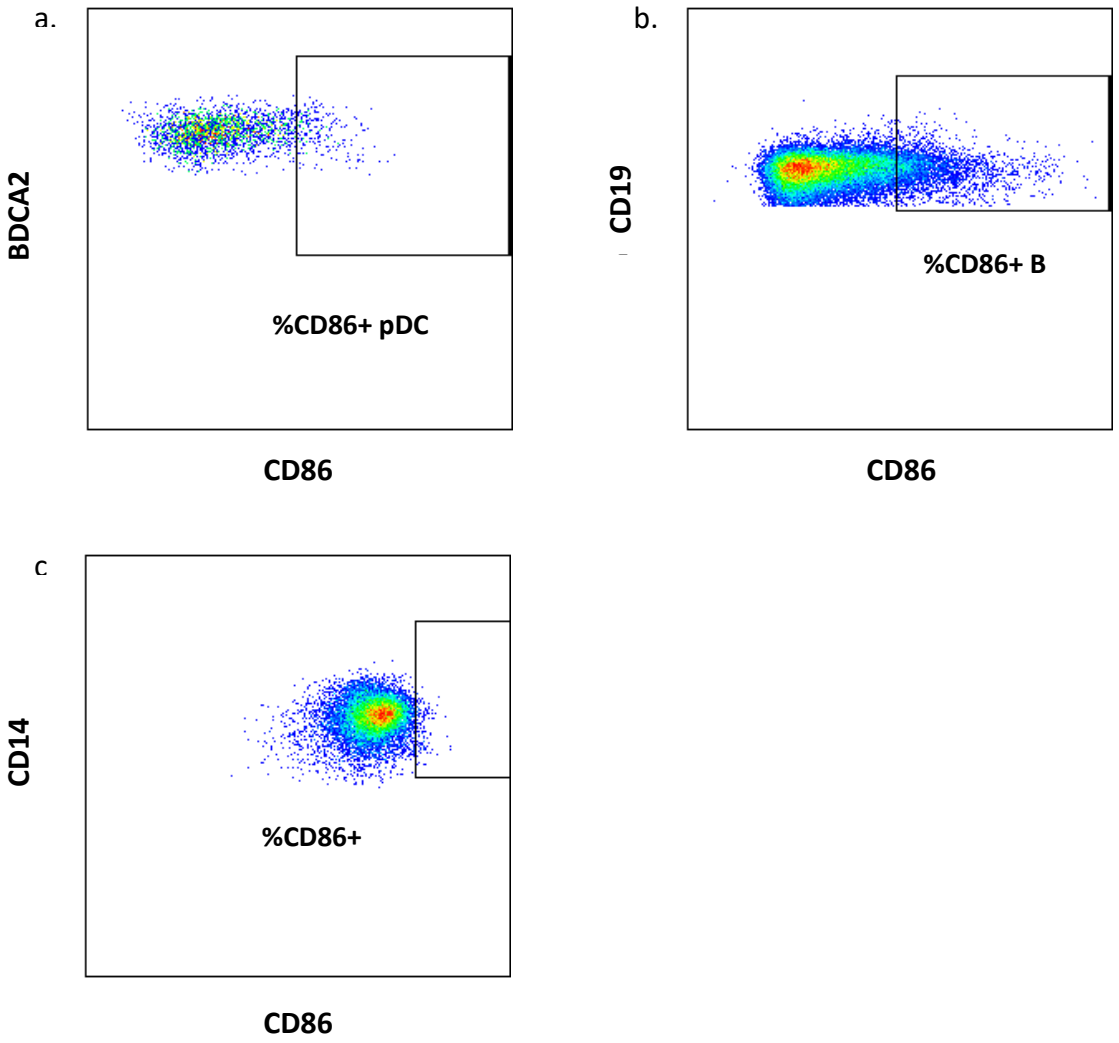


Figure 2-4. Flow cytometry measurement of CD86 expression in pDC, B cells and monocytes.

Gates were set using full minus one controls (not shown).

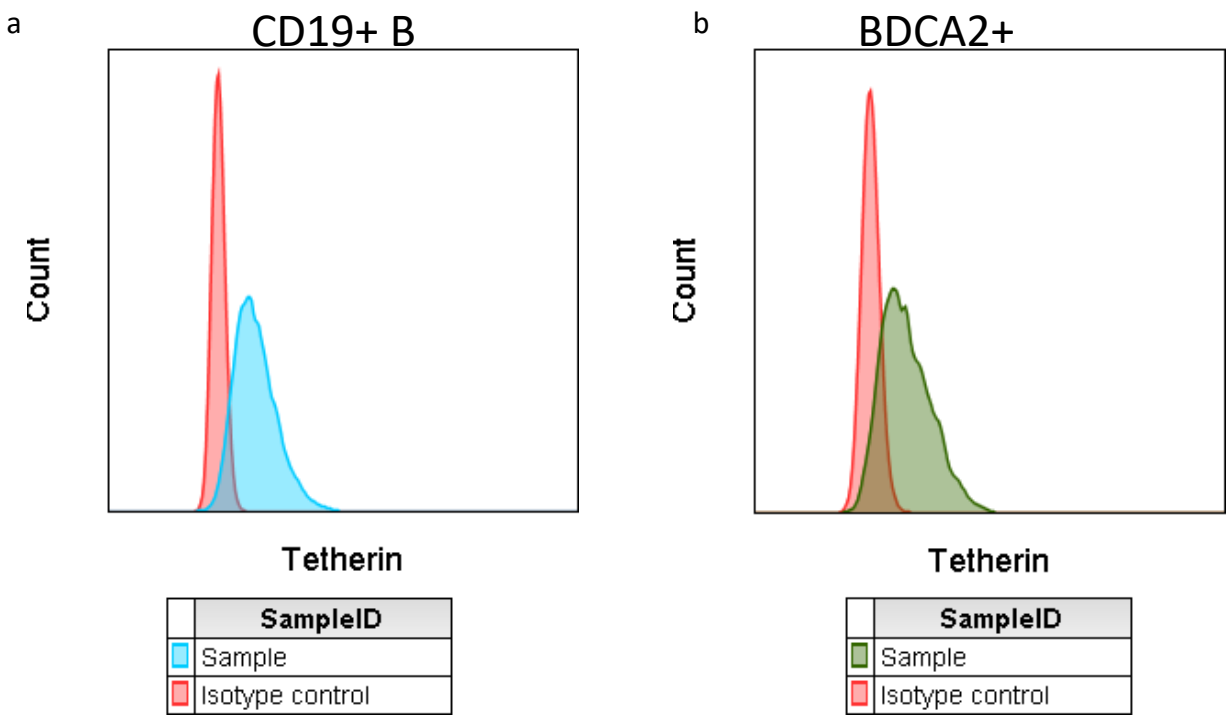


Figure 2-5. Tetherin expression. Tetherin was measured by flow cytometry, gates set using isotype controls as shown on a. B cells and b. pDC.

2.4.2. Post culture flow cytometry.

After culture with R848, CpG or no stimulation, cells were washed once with PBS, and re-suspended in a 50ul mixture of live/dead fixable stain near infrared (Biolegend, 423105) and the antibodies listed in Table 2-2 and incubated for 30 min in the dark at 4°C. Following incubation, cells were washed with PBS once before adding 100ul IC fix (Ebioscience IC fix 88-8824-00) and incubating in the dark at room temperature for 30 min. Thereafter cells were washed once with perm buffer before staining with antibody to IFNα (IFNα APC, Miltenyi Biotec, 130-092-602, clone: LT27:295) for 30 min in perm buffer. Cells were then washed once with FACS buffer before re-suspending in 100ul FACS buffer.

2.4.3. Flow cytometry analysis

Samples were run on an LSRII flow cytometer (BD) and the data were analysed using Flowjo v. 10. Gating strategy differed depending on whether PBMC were stimulated with CpG or R848. Upon CpG stimulation, there was a more pronounced downregulation of BDCA2 on pDC, requiring lymphocytes to be gated as lineage negative, CD123 positive, before gating on BDCA2 to gain the correct population of pDC. After R848 stimulation however, it was sufficient to gate pDC directly from lymphocytes as BDCA2 positive, CD123 positive (Fig. 2-6 and 2-7).

Antibody	Fluorochrome	Cat number	Clone	Company	Titre
Lineage - CD3, CD14, CD19, CD20, CD56	FITC	348701	UCHT1, HCD14, HIB19, 2H7, HCD56	Biolegend	1:200
BDCA2	PE	354203	201A	Biolegend	1:25
CD123	PECy7	306009	6H6	Biolegend	1:100

Table 2-2 Cultured cells surface staining panel.

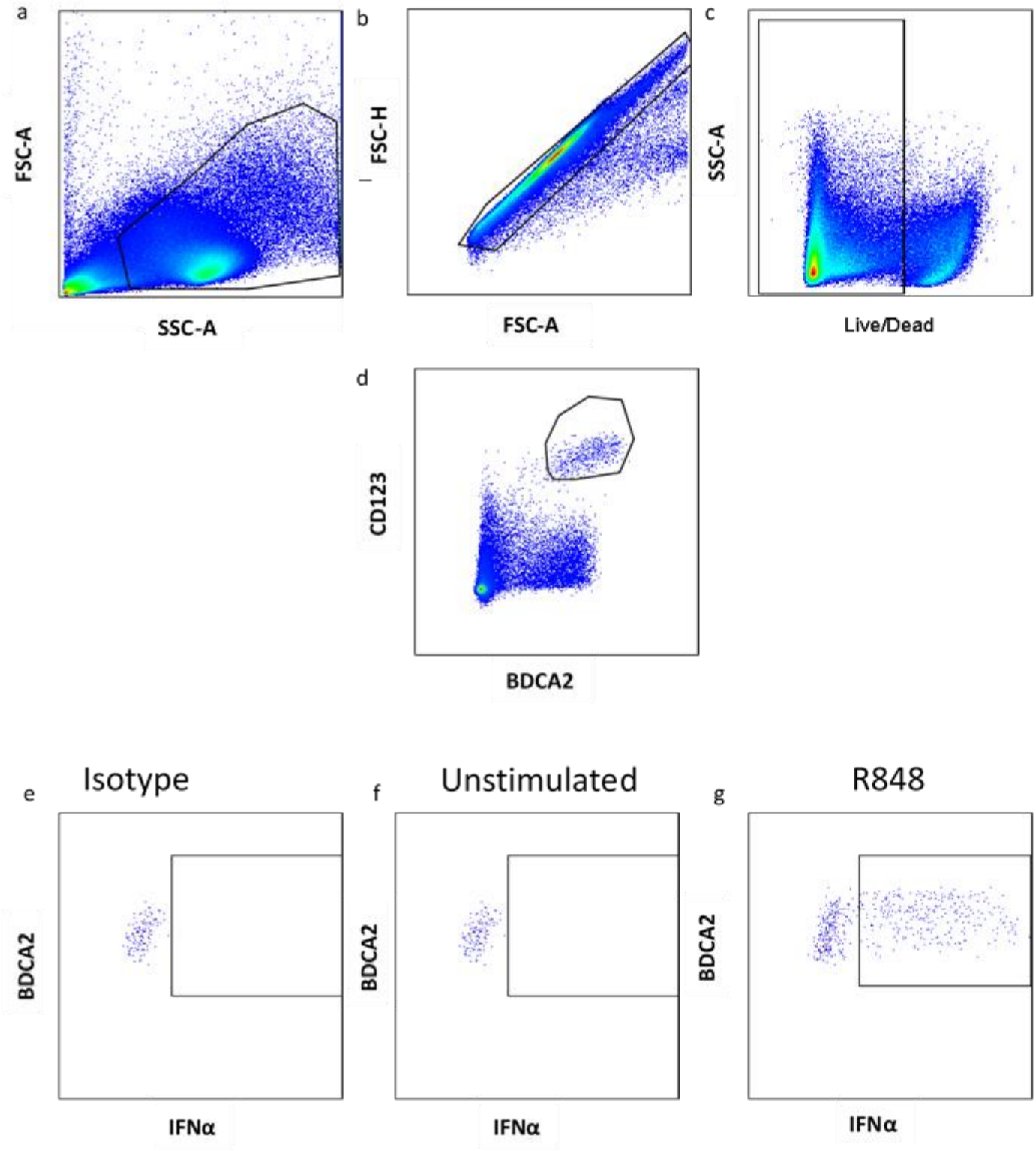


Figure 2-6. Flow cytometry gating strategy for R848 stimulated PBMC.PBMC were stimulated with R848. a.-b. By flow cytometry, lymphocytes and single cells were selected based on size. c. Live cells were selected. d. pDC were selected as CD123+; BDCA2+. e. An isotype control was used to set the gate for IFNα producing pDC and confirmed in f. unstimulated and g. R848 stimulated pDC.

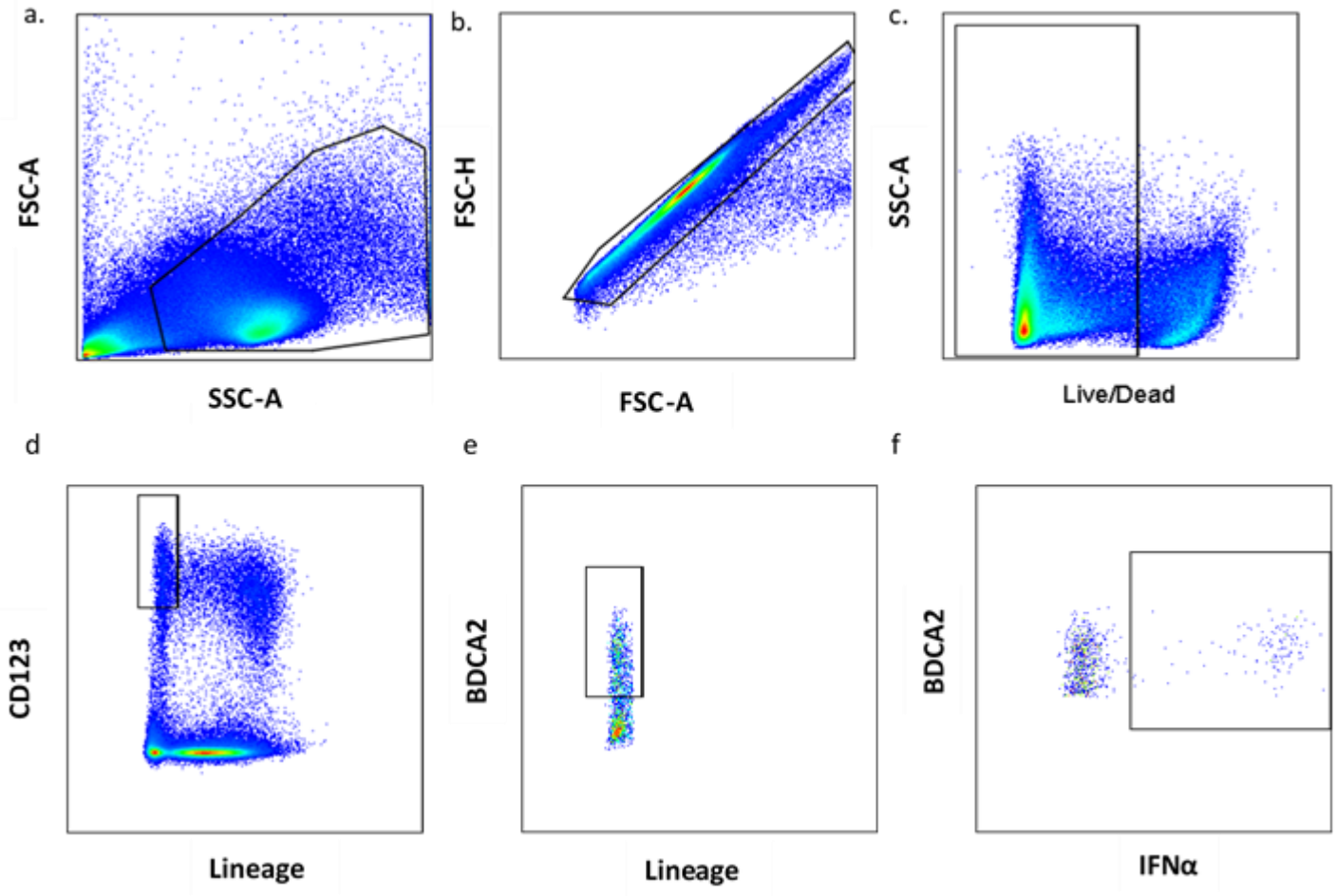


Figure 2-7. Flow cytometry gating strategy for CpG stimulated PBMC.PBMC were stimulated with CpG. a.-b. By flow cytometry, lymphocytes and single cells were gated based on size. c. Live cells were selected. d-e. CD123+; Lineage- cells were selected, before gating on BDCA2 to select pDC. f. The percentage of pDC producing IFNα was then assessed.

2.5. Cell culture supernatant assay.

2.5.1. Luminex

Separately, depending on cell availability, after thawing, PBMC were plated in a U bottomed 96 well plate at 1 million cells per well in media (RPMI+10%FCS). One million cells were each stimulated with TLR7 agonist, R848 (Invivogen-tlrl-r848) at 1ug/ml or TLR9 agonist, CpGODN2216 (CpG) (Invivogen tlrl-2216) at 1µM or left unstimulated (in RPMI+10%FCS) for 20 hours without the addition of BFA. After 20 hours, plates were centrifuged at 1800RPM for 3 minutes and supernatant was removed and cryopreserved at -80°C. Samples were transferred

to UMC Utrecht and strictly kept frozen on dry ice. UMC Utrecht multiplex facility has a ready optimised platform for the measurement of many cytokines, and a long term partnership with the UCL paediatric rheumatology unit(248, 249) which provided a cost-effective, efficient and reliable route for the analysis of these samples.Upon thawing, supernatants were analysed immediately by bead-based multiplex assay (Luminex-Thermofisher) at the UMC Utrecht facility for the concentrations of IFNα, IFNβ and TNFα (Fig 2-6). TNFα was chosen as a non-IFN related cytokine that is produced after TLR signalling to assess whether any response was IFN specific(16). Results that were reported as out of range were allocated to the maximum or minimum detectable levels represented below in Table 2-3. It was not possible to extrapolate out of this range and there was no further sample available to perform dilutions. The IFNα assay was reported by UMC Utrecht to detect all 13 subtypes of IFNα.

	Range of detection
TNFα	1.34-4962.42 pg./ml
IFNα	2.82-2499.70 pg./ml
IFNβ	4.28-13095.05 U/ml

Table 2-3. Range of detection as reported by UMC Utrecht.

These data were not normally distributed and the natural log of IFNα, IFNβ and TNFα concentration was used when these were analysed by linear regression.

2.3.2. ELISA

In early experiments optimising cell culture, supernatant was analysed by ELISA. Human IFN Alpha ELISA Kit (TCM) (PBL-411001) was used, according to manufacturer's instructions and reagents.

Briefly, all components were kept on ice and supernatant was thawed on nice. The exceptions were the wash solution and stop solution which were brought to room temperature. Standards were prepared with dilution buffer at the 156-5000pg/ml range or the 12.5-500pg/ml range as required. Samples were diluted in buffer 1:1 and plated in duplicate.100ul of sample/standard or blank was added to each well. The plate was covered with a plate sealer and incubated for 1 hour at room temperature. After 1 hour, the plate was emptied, and washed once with wash buffer. 100ul of antibody solution was added to each well, covered with plate sealer and incubated for 1 hour. After 1 hour, the plate was emptied and washed with wash buffer 3 times. 100ul of horse-radish protein (HRP) buffer was added to the wells and incubated for 1 hour. During this incubation trimethyl benzidine (TMB) substrate was brought to room temperature. After an hour, the plate was emptied and washed four times. 100ul of TMB substrate was added and plate was incubated in the dark for 15 min at room temp. After 15 min, stop solution was added to each well and the plate was read on a

microplate reader at 450nm within 5 min of adding stop solution. Optical densities were calculated using a 4-parameter fit for the standard curve and the IFNα concentration was determined.

2.6. RNA expression analysis.

2.6.1. IFNα stimulation.

Depending on availability, after thawing, PBMC were plated in a U bottomed 96 well plate at 1 million cells per well in media (RPMI+10%FCS) and washed once. One million cells were each stimulated with media (+10%FCS) containing 1000IU/ml IFNα2b (Sigma, SRP4595). After 20 hours, cells were washed once with media (RPMI+10%FCS) and RNA was extracted as below. Cells were incubated for 20 hours to coincide with the 20 hour stimulation of CpG and R848 as the experiments were all run together to optimise cell availability and experiment feasibility. A time point study was not performed.

2.6.2. RNA extraction.

Depending on availability, RNA was extracted from an aliquot of 1 million cells directly upon thawing or after 20 hours of stimulation with IFNα as above using the Picopure RNA isolation protocol and reagents from this kit (Thermofisher, KIT0214). An RNA free area was designated in the lab and RNAse was used in this area.

Briefly, cells were washed in suspension media (0.9ml 1XPBS; 10%FCS; 0.1ml 0.5M EDTA) and suspended in 100ul extraction buffer and incubated at 42°C for 30 minutes. Cells were thereafter centrifuged at 3000g before removing supernatant containing RNA and placing in a new, labelled 96 well plate and immediately freezing and storing at -80°.

Later, when needed, supernatant was thawed and 100ul of 70% ethanol was added to each well and mixed. Purification columns were pre-conditioned by adding 250ul of conditioning buffer to each column and leaving at room temperature for 5 minutes before centrifuging at 16000g for 1 minute. Samples plus ethanol were added to each purification column and micro-centrifuged at 100g for 2 minutes, followed by 1 minute at 16000g to bind to the filter. 100ul of wash buffer was added and spun at 8000g for 1 minute to remove sediment, this was repeated twice. The column was transferred to a new 0.5ml micro centrifuge tube and 11ul of elution buffer was directly pipetted onto each membrane. After 1-minute incubation at room temperature, each column was spun at 1000g for 1 minute flowed by 16000g for 1 minute to elute the RNA. RNA samples were assessed for amount and purity using Nanodrop technology and then labelled and stored at -80°C.

2.6.3. RNA analysis with Nanostring

24 genes were selected (including 3 housekeepers) and submitted to Nanostring (Table 2-4). Genes were selected to answer the original hypothesis:

-Known IFN inducible genes to construct an IFN signature (*MX1, BST2, IFIT1, ISG15, MCP1*)

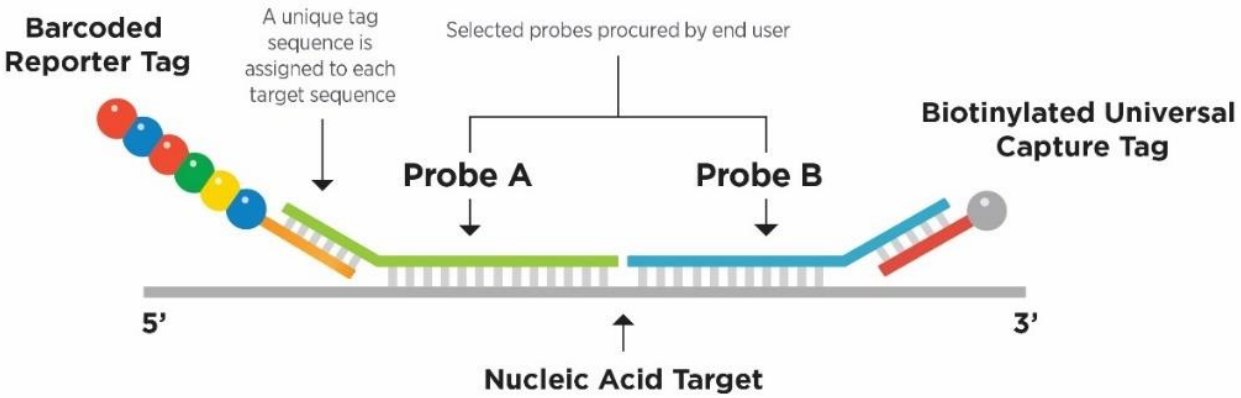
-Genes in the TLR signalling pathway (*TLR7/9/IRF5/7*)

-Possible DNA/RNA ligand source, cell death genes (*LINE1, TNFR6, TRAIL, Ro60*)

-Intracytoplasmic RNA /DNA sensors (*MDA5, MAVS, DDx41, RIG-1*)

-Hormone receptor genes.

Nanostring then designed two cDNA probes for each gene (probe A and probe B) which were manufactured by Integrated DNA Technologies (IDT, Coralville, IA). Probe A and B each contain gene-specific sequence; probe A also has a gene-specific sequence that binds the specific barcoded reporter tag, whereas probe B binds to a universal capture tag (Figure 2-8). Nucleotide sequences for probes A and B are listed in Table 2-5 and 2-6.



L1	*LINE-1*	*Endogenous retroelement*
DDX41	*Dead box helicase 41*	*cytoplasmic viral sensing*
MDA5	*Melanoma differentiated protein 5*	*cytoplasmic viral sensing*
RIG1	*Retinoid inducible gene 1*	*cytoplasmic viral sensing*
MAVS	*Mitochondrial antiviral signalling protein*	*cytoplasmic viral sensing*
TROVE2	*Ro60*	*Ro 60*
ESR1	*estrogen receptor alpha*	*hormone receptor*
ESR2	*estrogen receptor beta*	*hormone receptor*
AR	*androgen receptor*	*hormone receptor*
PGR	*progesterone receptor*	*hormone receptor*
G6PD	*housekeeper*	
POL2RA	*housekeeper*	
SDHA	*housekeeper*	

. Figure 2-8. Nanostring technology (from Nanostring). NanoString technology uses synthetic complementary DNA oligonucleotides to directly bind, capture, and tag RNA molecules of interest for fluorescent field counting without reverse transcription or amplification.

We further extended this technology and plexed multiple samples. Nanostring designed a PlexSet, with a unique mix of barcodes for each row of a 96 well plate. This allows the pooling of multiple samples in a column, allowing a single run to assess multiple samples (Fig. 2-9).

Samples were hybridised in individual wells at 67°C for 24 h, and then pooled. Pooled samples were then processed using the "High Sensitivity" protocol option on the nCounter Prep Station and counted on nCounter Digital Analyzer using maximal data resolution. Data were processed with nSolver software (NanoString Technologies, Seattle, WA) which includes quality analysis

of the runs. Counts are normalised via six internal positive control sequences and predetermined housekeeping genes (*SDHA, G6PD* and *POL2RA*). These normalized counts are used for downstream analysis.

A calibration lane was spiked with synthetic manufactured RNA oligonucleotides in order to control for variation between PlexSets.

Nanostring and specifically PlexSet technology has been used before in the study of ISG and shown to be reliable and reproducible(250) (251).

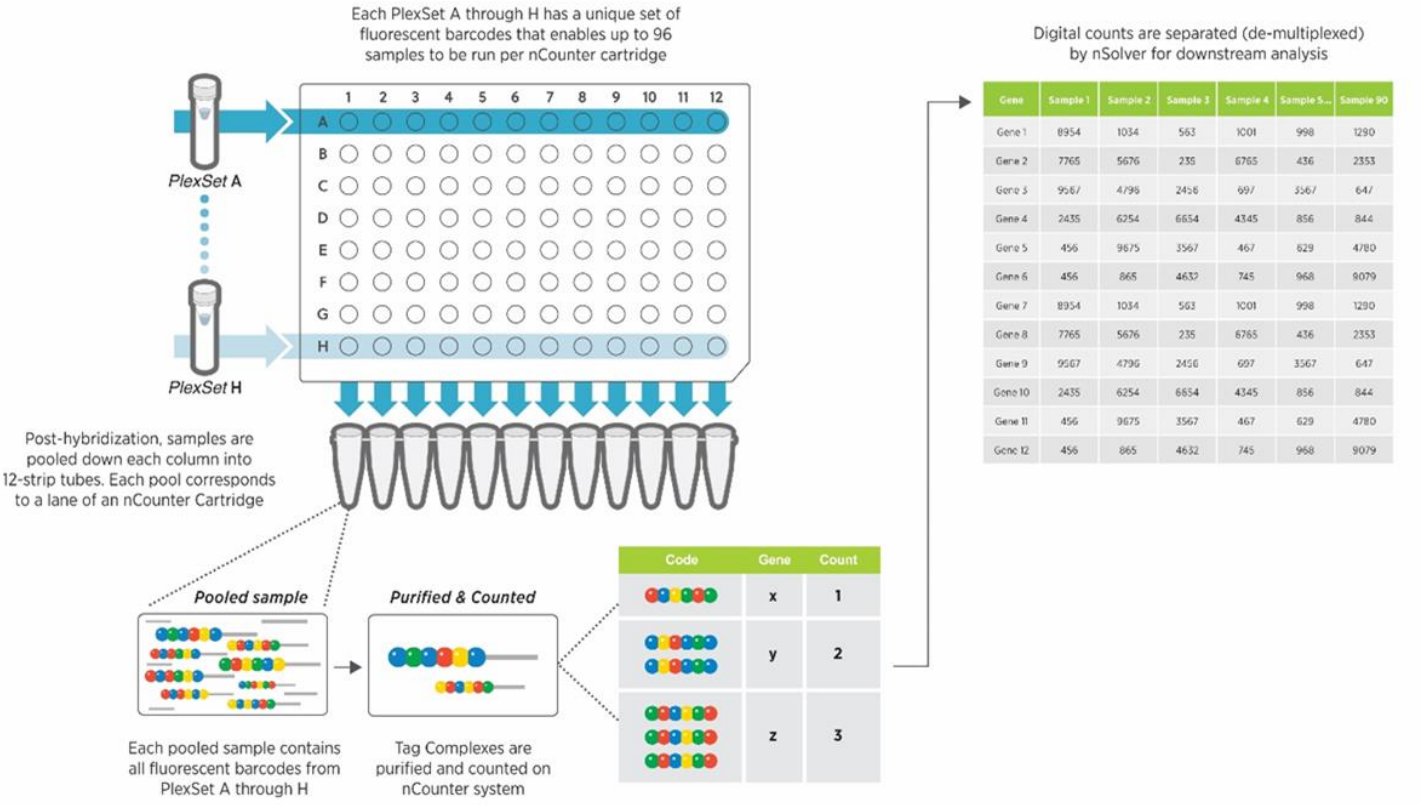


Figure 2-7. Plexset technology (From Nanostring). PlexSet allows the pooling of multiple samples to be analysed in a single run.

Gene number	Gene name	Probe A sequence
NM_002462	MX1	CCTACAGTTTCCTCTCCTTGCATGAGAGCAGTGATGTCCTGATTAAAGGCCCTCAAGACCTAAGCGACAGCGTGACCTTGTTTCA
NM_004335	BST2	TACAGCGCTTATCCCCGTCTTCCATGGGCACTCTGCATCCTCTTCTTTTCTTGGTGTTGAGAAGATGCTC
NM_001548	IFIT1	TGTAGACGAACCCAAGGAGGCTCAAGCTTTCCAGATCTAATGCCTTTCTCCACAATTCTGCGGGTTAGCAGGAAGGTTAGGGAAC
NM_005101	ISG15	CTCAGAGGTTCGTCGCATTTGTCCACCACCAGCAGGACCTGTTGAGATTATTGAGCTTCATCATGACCAGAAG
NM_002982	MCP1	CAGTTTGAGAATTGGATGTTTCTGGGTTAGTCTCAGCCTCTCGGTTCCTCCAAAGACGCCTATCTTCCAGTTTGATCGGGAAACT
NM_002200	IRF5	GTTGGGAGAGTTCTTTCCCTGCTCATGGCTGAATTTCCCGAGAGCCAGCGAACCTAACTCCTCGCTACATTCCTATTGTTTTC
NM_004029	IRF7	TCGTCATAGAGGCTGTTGGCGCTGGACAGGCAGAGCCAATTTGGTTTTACTCCCCTCGATTATGCGGAGT
NM_016562	TLR7	GAAAAAGAGCAGGAAGCACTGAAGCAGCAACCACCTGTGTGGTGCCCACACTTTCGGGTTATATCTATCATTTACTTGACACCCT
NM_017442	TLR9	TTGTTGTAGCTCAGGTTTAGCTCTTCCAGGGTGGGCACAGCAACAGCCACTTTTTTTCCAAATTTTGCAAGAGCC
NM_000043	TNFR6	CATTCTTGATCTCATCTATTTTGGCTTCATTGACACCATTCTTTCGAACACACCGTGTGGACGGCAACTCAGAGATAACGCATAT
NM_003810	TRAIL	CTGTGAAGATCACGATCAGCACGCAGGTCTGTCCCCCTGGAGTTTATGTATTGCCAACGAGTTTGTCTTT
U09116	LINE1	CTCTGCACATGAGATGGGTTTCCTGAATACAGCACACTGATGGGTCTTGACAGATAAGGTTGTTATTGTGGAGGATGTTACTACA
NM_016222	DDX41	ATGACGGGCAACGTGAACACCAGTGTCTTGCCTGAACCCGTGAAAGCGATCTTCCTTCCTGTGTTCCAGCTACAAACTTAGAAAC
NM_022168	MDA5	CTTCTCCAGATTTGGCTGAACTGTGGTTGAAAGAAGTTGCTCTTCCACTTCATAAAATTGGTTTTGCCTTTCAGCAATTCAACTT
NM_014314	RIG1	GGTCATTCCTGTGTTCTGATTTGTTTTGCCACGTCCAGTCAATATGCCAGCTGGTCAAGACTTGCATGAGGACCCGCAAATTCCT
NM_020746	MAVS	ACCCCAATGTGTTTCTGGTGTTATTGTAGATAATCTCCCCAACAGTGGGTCTTTCGTTGGGACGCTTGAAGCGCAAGTAGAAAAC
NM_001042370	TROVE2	CCTCCAGCAAAGGTCTCATTATCAGTGAATACAATGAAGACATCAGCAGGCCAGCAGACCTGCAATATCAAAGTTATAAGCGCGT
NM_000125	ESR1	AGCATGTCGAAGATCTCCACCATGCCCTCTACACATTTTCCCTGGTTCCTGCCAATGCACTCGATCTTGTCATTTTTTTGCG
NM_001214903	ESR2	TTCATGGTGGCTGTCTACATAGGAGGAAGGTATGTATATGGAGCCGTGCAAACTGGAGAGAGAAGTGAAGACGATTTAACCCA
NM_000044	AR	CAGAAGAAGACACACGGTGGACAAGATCTGCCCTGCTAGGCTCACAGCGATTGCTGCATTCCGCTCAACGCTTGAGGAAGTA
NM_000926	PGR	AAATCTTCTGAGGTAATGACTCGAAGCTGTATTGTGGGCTCTGGCTGGCTCTGAGGCTGTTAAAGCTGTAGCAACTCTTCCACGA
NM_000937	G6PD	ACTGGCCCAACAGGAAGACAGTAAGCGAAGGAGTCTTTGGCTTCTTGGAACTAGGACGCAAATCACTTGAAGAAGTGAAAGCGAG
NM_000402	POL2RA	CTCAGTGCCAAAGGGCTCCTTGAAGGTGAGGATAACGCAGGCGATGTTGTCCACGCGATGACGTTCGTCAAGAGTCGCATAATCT
NM_004168	SDHA	TAAACCCTGCCTCAGAAAGGCCAAATGCAGCTCGCAAGCCTGCCATTTGGAATGATGTGTACTGGGAATAAGACGACG

Table 2-5. Probe A sequence design

Gene number	Gene name	Probe B sequence
NM_002462	MX1	CCTACAGTTTCCTCTCCTTGCATGAGAGCAGTGATGTCCTGATTAAAGGCCCTCAAGACCTAAGCGACAGCGTGACCTTGTTTCA
NM_004335	BST2	TACAGCGCTTATCCCCGTCTTCCATGGGCACTCTGCATCCTCTTCTTTTCTTGGTGTTGAGAAGATGCTC
NM_001548	IFIT1	TGTAGACGAACCCAAGGAGGCTCAAGCTTTCCAGATCTAATGCCTTTCTCCACAATTCTGCGGGTTAGCAGGAAGGTTAGGGAAC
NM_005101	ISG15	CTCAGAGGTTCGTCGCATTTGTCCACCACCAGCAGGACCTGTTGAGATTATTGAGCTTCATCATGACCAGAAG
NM_002982	MCP1	CAGTTTGAGAATTGGATGTTTCTGGGTTAGTCTCAGCCTCTCGGTTCCTCCAAAGACGCCTATCTTCCAGTTTGATCGGGAAACT
NM_002200	IRF5	GTTGGGAGAGTTCTTTCCCTGCTCATGGCTGAATTTCCCGAGAGCCAGCGAACCTAACTCCTCGCTACATTCCTATTGTTTTC
NM_004029	IRF7	TCGTCATAGAGGCTGTTGGCGCTGGACAGGCAGAGCCAATTTGGTTTTACTCCCCTCGATTATGCGGAGT
NM_016562	TLR7	GAAAAAGAGCAGGAAGCACTGAAGCAGCAACCACCTGTGTGGTGCCCACACTTTCGGGTTATATCTATCATTTACTTGACACCCT
NM_017442	TLR9	TTGTTGTAGCTCAGGTTTAGCTCTTCCAGGGTGGGCACAGCAACAGCCACTTTTTTTCCAAATTTTGCAAGAGCC
NM_000043	TNFR6	CATTCTTGATCTCATCTATTTTGGCTTCATTGACACCATTCTTTCGAACACACCGTGTGGACGGCAACTCAGAGATAACGCATAT
NM_003810	TRAIL	CTGTGAAGATCACGATCAGCACGCAGGTCTGTCCCCCTGGAGTTTATGTATTGCCAACGAGTTTGTCTTT
U09116	LINE1	CTCTGCACATGAGATGGGTTTCCTGAATACAGCACACTGATGGGTCTTGACAGATAAGGTTGTTATTGTGGAGGATGTTACTACA
NM_016222	DDX41	ATGACGGGCAACGTGAACACCAGTGTCTTGCCTGAACCCGTGAAAGCGATCTTCCTTCCTGTGTTCCAGCTACAAACTTAGAAAC
NM_022168	MDA5	CTTCTCCAGATTTGGCTGAACTGTGGTTGAAAGAAGTTGCTCTTCCACTTCATAAAATTGGTTTTGCCTTTCAGCAATTCAACTT
NM_014314	RIG1	GGTCATTCCTGTGTTCTGATTTGTTTTGCCACGTCCAGTCAATATGCCAGCTGGTCAAGACTTGCATGAGGACCCGCAAATTCCT
NM_020746	MAVS	ACCCCAATGTGTTTCTGGTGTTATTGTAGATAATCTCCCCAACAGTGGGTCTTTCGTTGGGACGCTTGAAGCGCAAGTAGAAAAC
NM_001042370	TROVE2	CCTCCAGCAAAGGTCTCATTATCAGTGAATACAATGAAGACATCAGCAGGCCAGCAGACCTGCAATATCAAAGTTATAAGCGCGT
NM_000125	ESR1	AGCATGTCGAAGATCTCCACCATGCCCTCTACACATTTTCCCTGGTTCCTGCCAATGCACTCGATCTTGTCATTTTTTTGCG
NM_001214903	ESR2	TTCATGGTGGCTGTCTACATAGGAGGAAGGTATGTATATGGAGCCGTGCAAACTGGAGAGAGAAGTGAAGACGATTTAACCCA
NM_000044	AR	CAGAAGAAGACACACGGTGGACAAGATCTGCCCTGCTAGGCTCACAGCGATTGCTGCATTCCGCTCAACGCTTGAGGAAGTA
NM_000926	PGR	AAATCTTCTGAGGTAATGACTCGAAGCTGTATTGTGGGCTCTGGCTGGCTCTGAGGCTGTTAAAGCTGTAGCAACTCTTCCACGA
NM_000937	G6PD	ACTGGCCCAACAGGAAGACAGTAAGCGAAGGAGTCTTTGGCTTCTTGGAACTAGGACGCAAATCACTTGAAGAAGTGAAAGCGAG
NM_000402	POL2RA	CTCAGTGCCAAAGGGCTCCTTGAAGGTGAGGATAACGCAGGCGATGTTGTCCACGCGATGACGTTCGTCAAGAGTCGCATAATCT
NM_004168	SDHA	TAAACCCTGCCTCAGAAAGGCCAAATGCAGCTCGCAAGCCTGCCATTTGGAATGATGTGTACTGGGAATAAGACGACG

Table 2-6. Probe B sequence design

2.6.3.1. Nanostring optimisation

A titration experiment was performed with the aims of optimising

1) Specific gene detection

2) Sample input optimisation

3) Ensuring that maximum saturation and data loss did not occur when plexing samples

RNA from 2 representative samples was used from healthy teenagers:

T161-ex vivo RNA

T 139-Stimulated with IFNα for 20 hours

3 different input amounts of RNA was used from each subject in duplicate (20ng/100ng/300ng).

RNA was hybridised with oligonucleotide probe sets and unique reporter and capture tags. These were then scanned using a nCounter Flex. Inbuilt positive controls were used to normalise each lane. (Fig. 2-10).

All genes had acceptable expression at 300ng input in stimulated and unstimulated samples except for TLR7,9, AR, ESR1, ESR2 and PGR (Fig. 2-11). Among these genes, TLR7 and 9 had low expression but good correlation between sample input and count number implying real expression above background (R^2=0.998 for both). Relatively poor correlation among the steroid receptors implies that these may not be expressed in these cell populations at a level high enough for accurate quantification (R^2<0.982 for all). Therefore increasing the sample input would improve detection of TLRs but not necessarily hormone receptors.

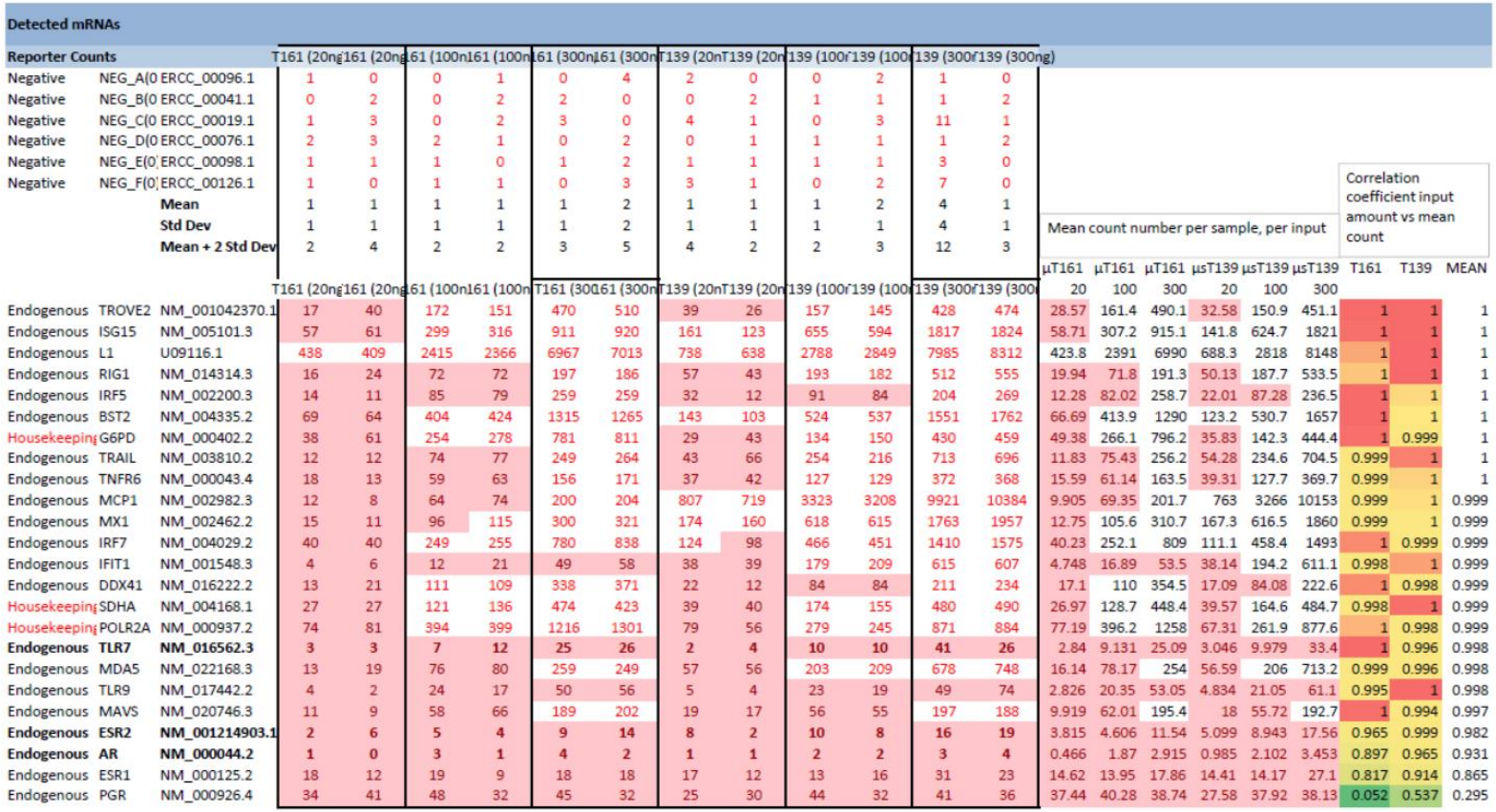
In the final plexset experiment, 8 samples are pooled. It is important to ensure that the counts anticipated per sample do not exceed 150000 counts (8x150000 in total after pooling) which is the saturation limit for the nCounter Flex system. Maximum sample inputs (T161=2919.56ng; T139=1458.49ng) were calculated for each representative sample in this experiment (Fig. 2-12), which far exceed the current input of 300-500ng. In addition there is a trend towards plateau in detection at 300ng input amount in overall detection, and counts only reach 10% of the saturation limit.

Therefore, all genes were expressed at acceptable levels, except for TLR7/9 and the steroid receptors. Increasing sample input to 500ng in certain samples where available should increase

detectability of TLR7/9 but not necessarily hormone receptors. In addition, detectability is reasonable at these concentrations and there is very little risk of saturation.

Reporter Counts														
Code Class	**Name**	**Accession**												
Positive	POS_A(128	ERCC_0003	65345	63517	64124	68247	62133	64343	68302	56617	57302	59005	45119	58004	
Positive	**POS_B(32)**	**ERCC_0011**	**9649**	**9326**	**9574**	**9943**	**9003**	**9335**	**9938**	**8254**	**8289**	**8501**	**6551**	**8511**	
Positive	POS_C(8)	ERCC_0006	3870	3745	3821	4060	3693	3902	4088	3435	3406	3413	2735	3442	
Positive	POS_D(2)	ERCC_0009	447	418	469	481	403	474	423	413	398	423	329	408	
Positive	POS_E(0.5)	ERCC_0003	163	132	164	158	134	125	172	169	150	141	118	153	
Positive	POS_F(0.1)	ERCC_0011	54	47	59	47	44	53	40	31	39	60	43	51	
			0.98931	0.98918	0.98962	0.98889	0.98874	0.9887	0.98885	0.98882	0.98865	0.98858	0.98869	0.98909	
	Sum		**79528**	**77185**	**78211**	**82936**	**75410**	**78232**	**82963**	**68919**	**69584**	**71543**	**54895**	**70569**	
	Average														**74165**
	Lane normalisation fa		0.933	0.961	0.948	0.894	0.983	0.948	0.894	1.076	1.066	1.037	1.351	1.051	

Figure 2-10: Normalisation of RNA data. Inbuilt positive controls were used to normalise counts across lanes. Normalisation factor is calculate by dividing average counts across all lanes by individual lane count. Sample counts are then corrected by lane normalisation factor



Detected mRNAs																							
Reporter Counts			T161 (20ng	161 (20ng	61 (100n	161 (100n	161 (300n	161 (300n	T139 (20n	T139 (20n	139 (100r	139 (100r	139 (300r	139 (300ng)									
Negative	NEG_A(0	ERCC_00096.1	1	0	0	1	0	4	2	0	0	2	1	0									
Negative	NEG_B(0	ERCC_00041.1	0	2	0	2	2	0	0	2	1	1	1	2									
Negative	NEG_C(0	ERCC_00019.1	1	3	0	2	3	0	4	1	0	3	11	1									
Negative	NEG_D(0	ERCC_00076.1	2	3	2	1	0	2	0	1	1	1	1	2									
Negative	NEG_E(0	ERCC_00098.1	1	1	1	0	1	2	1	1	1	1	3	0									
Negative	NEG_F(0	ERCC_00126.1	1	0	1	1	0	3	3	1	0	2	7	0									
		Mean	1	1	1	1	1	2	1	1	1	2	4	1									
		Std Dev	1	1	1	1	1	2	1	1	1	1	4	1									
		Mean + 2 Std Dev	2	4	2	2	3	5	4	2	2	3	12	3									
			T161 (20ng	161 (20ng	61 (100n	161 (100n	T161 (300	161 (300n	T139 (20n	T139 (20n	139 (100r	139 (100r	139 (300r	139 (300	Mean count number per sample, per input						Correlation coefficient input amount vs mean count		
															µT161	µT161	µT161	µsT139	µsT139	µsT139	T161	T139	MEAN
															20	100	300	20	100	300			
Endogenous	TROVE2	NM_001042370.1	17	40	172	151	470	510	39	26	157	145	428	474	28.57	161.4	490.1	32.58	150.9	451.1	1	1	1
Endogenous	ISG15	NM_005101.3	57	61	299	316	911	920	161	123	655	594	1817	1824	58.71	307.2	915.1	141.8	624.7	1821	1	1	1
Endogenous	L1	U09116.1	438	409	2415	2366	6967	7013	738	638	2788	2849	7985	8312	423.8	2391	6990	688.3	2818	8148	1	1	1
Endogenous	RIG1	NM_014314.3	16	24	72	72	197	186	57	43	193	182	512	555	19.94	71.8	191.3	50.13	187.7	533.5	1	1	1
Endogenous	IRF5	NM_002200.3	14	11	85	79	259	259	32	12	91	84	204	269	12.28	82.02	258.7	22.01	87.28	236.5	1	1	1
Endogenous	BST2	NM_004335.2	69	64	404	424	1315	1265	143	103	524	537	1551	1762	66.69	413.9	1290	123.2	530.7	1657	1	1	1
Housekeeping	G6PD	NM_000402.2	38	61	254	278	781	811	29	43	134	150	430	459	49.38	266.1	796.2	35.83	142.3	444.4	1	0.999	1
Endogenous	TRAIL	NM_003810.2	12	12	74	77	249	264	43	66	254	216	713	696	11.83	75.43	256.2	54.28	234.6	704.5	0.999	1	1
Endogenous	TNFR6	NM_000043.4	18	13	59	63	156	171	37	42	127	129	372	368	15.59	61.14	163.5	39.31	127.7	369.7	0.999	1	1
Endogenous	MCP1	NM_002982.3	12	8	64	74	200	204	807	719	3323	3208	9921	10384	9.905	69.35	201.7	763	3266	10153	0.999	1	0.999
Endogenous	MX1	NM_002462.2	15	11	96	115	300	321	174	160	618	615	1763	1957	12.75	105.6	310.7	167.3	616.5	1860	0.999	1	0.999
Endogenous	IRF7	NM_004029.2	40	40	249	255	780	838	124	98	466	451	1410	1575	40.23	252.1	809	111.1	458.4	1493	1	0.999	0.999
Endogenous	IFIT1	NM_001548.3	4	6	12	21	49	58	38	39	179	209	615	607	4.748	16.89	53.5	38.14	194.2	611.1	0.998	1	0.999
Endogenous	DDX41	NM_016222.2	13	21	111	109	338	371	22	12	84	84	211	234	17.1	110	354.5	17.09	84.08	222.6	1	0.998	0.999
Housekeeping	SDHA	NM_004168.1	27	27	121	136	474	423	39	40	174	155	480	490	26.97	128.7	448.4	39.57	164.6	484.7	0.998	1	0.999
Housekeeping	POLR2A	NM_000937.2	74	81	394	399	1216	1301	79	56	279	245	871	884	77.19	396.2	1258	67.31	261.9	877.6	1	0.998	0.999
Endogenous	**TLR7**	**NM_016562.3**	**3**	**3**	**7**	**12**	**25**	**26**	**2**	**4**	**10**	**10**	**41**	**26**	2.84	9.131	25.09	3.046	9.979	33.4	1	0.996	0.998
Endogenous	MDA5	NM_022168.3	13	19	76	80	259	249	57	56	203	209	678	748	16.14	78.17	254	56.59	206	713.2	0.999	0.996	0.998
Endogenous	TLR9	NM_017442.2	4	2	24	17	50	56	5	4	23	19	49	74	2.826	20.35	53.05	4.834	21.05	61.1	0.995	1	0.998
Endogenous	MAVS	NM_020746.3	11	9	58	66	189	202	19	17	56	55	197	188	9.919	62.01	195.4	18	55.72	192.7	1	0.994	0.997
Endogenous	**ESR2**	**NM_001214903.1**	**2**	**6**	**5**	**4**	**9**	**14**	**8**	**2**	**10**	**8**	**16**	**19**	3.815	4.606	11.54	5.099	8.943	17.56	0.965	0.999	0.982
Endogenous	**AR**	**NM_000044.2**	**1**	**0**	**3**	**1**	**4**	**2**	**1**	**1**	**2**	**2**	**3**	**4**	0.466	1.87	2.915	0.985	2.102	3.453	0.897	0.965	0.931
Endogenous	ESR1	NM_000125.2	18	12	19	9	18	18	17	12	13	16	31	23	14.62	13.95	17.86	14.41	14.17	27.1	0.817	0.914	0.865
Endogenous	PGR	NM_000926.4	34	41	48	32	45	32	25	30	44	32	41	36	37.44	40.28	38.74	27.58	37.92	38.13	0.052	0.537	0.295

Figure 2-9: Lane normalised counts of RNA in 600 fields of view. All negative controls showed low mean background counts. Counts highlighted in pink show poor detection (using 100 counts as an arbitrary low count number). Mean count number was assessed per input amount per sample. A correlation was performed to assess the correlation between input amount and count number. Even though TLR7 and 9 had low expression, there was reasonable correlation (r^2=0.998 for each), suggesting that the gene is detectable. For the hormone receptors (ESR2, AR, ESR1 and PGR) there is a relatively poor correlation, implying less reliable detection of these genes. Therefore, an input higher than 300ng where possible would improve the detection of the low expressed genes, but not necessarily the hormone receptors.

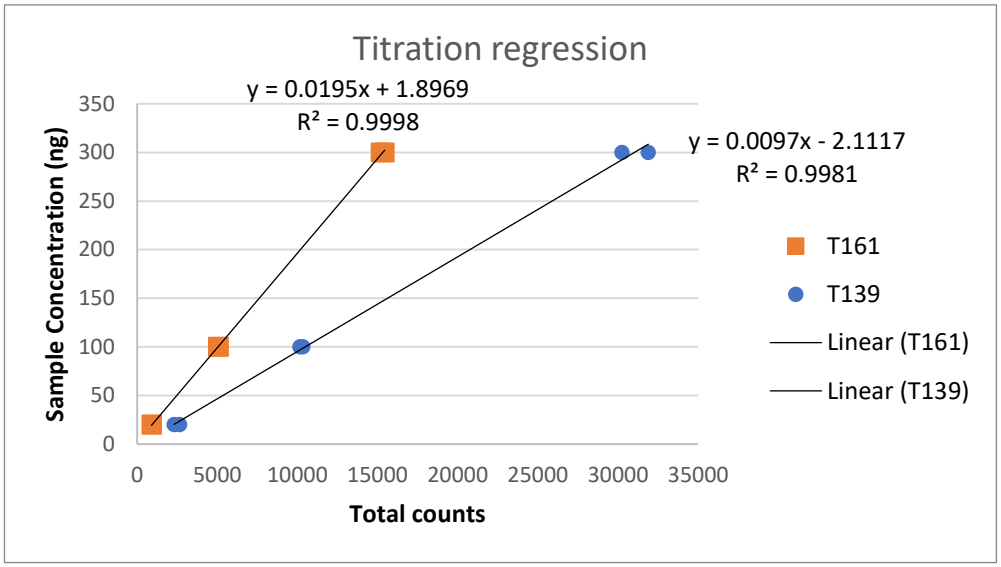


Figure 2-12: Linear regression was used to estimate equations to predict the maximum sample volume that would exceed 150000 counts. T161-Max sample input =0.0195x150000+1.8969, therefore max sample input= 2919.56377ng. T139-Max sample input =0.0097x150000+2.1117 therefore max sample input= 1458.49435ng

Therefore, although counts were low, TLR7 and TLR9 expression was reliably measured. This was not the case for the steroid receptors (oestrogen, progesterone and androgen receptor) whose detection was low, along with a poor correlation with input amounts, implying an imprecise measurement. The steroid receptor data was therefore excluded from further analysis. Samples with at least 300ng RNA with adequate purity (260/280>1.8 and 260/230>1.4) were prepared for analysis.

2.7. Liquid chromatography/Mass spectrometry.

Serum sex steroid analysis was performed using clinically optimised liquid chromatography and tandem mass spectrometry (LC/MS) at the Biochemistry Department at the Wythenshawe Hospital, Manchester University NHS Foundation Trust. Briefly, LC/MS is a technique that combines the physical separation capabilities of high performance liquid chromatography with the mass analysis capabilities of mass spectrometry. The liquid chromatography effectively separates mixtures with multiple components, and the mass spectrometry structurally identifies the individual components with high specificity and sensitivity.

Local clinical and commercially available measurement of sex steroid hormones is performed with radio or fluorescent based immunoassays. These do not detect serum steroid hormones at low concentrations, for example in young children, and are less specific than LC/MS (252) (253).

The lower detection limits of serum oestradiol using this assay was 10pmol/L, and serum testosterone was 0.1nmol/L.

2.8. Statistical Analysis.

Data were analysed using linear regression, t-tests or Mann Whitney U tests as statistically appropriate. Where necessary, the Benjamini Hochberg or Bonferroni methods of correction for multiple hypothesis testing were applied. SPSS software was used for statistical analysis (v.24). Graphs were created with SPSS or Biovinci graph software (v.1.3). If outliers were present, it was tested if their effect was influential or not. If not found to be influential, it was elected to keep them in the analysis. If it was elected to exclude them, it is reported in the results chapters below.

In linear regression, a model is built to predict the variability of the outcome variable (x), by using input variables ($y_{1;}$ $y_{2...}$.etc). This builds a model like this:

$$X = B_{1.}\, Intercept + B_2.y_1 + B_2.Y_2 \ldots$$

The B values represent the coefficients of the predictor variables (y_1, y_2 etc). They essentially represent an equation, where, for every change of 1 in y_1, there is a subsequent change of B in x, keeping the other predictor variables constant. If a predictor variable is binary (e.g. sex) then it is coded 0 or 1. Therefore the B coefficient estimates the predicted change in the outcome variable between sexes, holding the other variables in the equation constant. The adjusted r^2 value indicates the amount of variation in the outcome variable (x) that can be predicted by all

of the predictor variables (y_1, y_2, etc) in the model. Essentially it shows how 'good' the model is at accounting for the outcome variable of interest. In addition, the adjusted r^2 estimates the most parsimonious model. This means that the model with the best predictive value, using the fewest predictor values is developed.

In these data, there were many variables that were being analysed. The main predictors were sex, puberty, X chromosome number, serum testosterone and oestradiol concentrations. These variables required sophisticated modelling in order to assess the true effect of one, while correcting for the effects of the others in the equations. Linear regression models with more than 2 predictor variables cannot be represented by a 2 dimensional graph, however it was important to visually represent these data. Therefore when two variables are represented in this thesis, it is with the caveat that this is an illustration of the raw data only, and the p values represent the coefficient of the variable shown, holding the other variables in the equation constant. In addition, the raw data is represented on the graph, but the p vaue for the coefficient of the variable shown actually represents data that has been corrected for the other variables in the model. Therefore, it is actually impossible to accurately graphically represent complex models such as these, but the data is shown here and the models have been represented as best as they can be on the graphs for the purpose of clarity and probity.

When other statistical tests are used, (t-tests, ANOVA , correlation) these are specifically mentioned.

Chapter 3: Summary of participants

Participants were recruited as outlined above. In this chapter, key demographic and clinical characteristics of the participants that will be relevant to the subsequent main results chapters 4-7 will be outlined. In addition, the model that will be used in the main results chapters to individually evaluate the contributions of serum sex hormone concentration and X chromosome number on the measured immune parameters in this thesis will be introduced.

3.1. Healthy volunteers

One hundred healthy volunteers were recruited. One 13 year old female was excluded due to a serum oestradiol concentration of 2106pmol/L which is outside of the range of normal for this age. The ages and ethnicity of the rest of the volunteers are summarised in Tables 3-1 and 3-2.

				Age			
			n	Mean	Median	Min	Max
Female	Pubertal phase	Pre-pubertal	20	8.8	8.7	6.3	12.01
		Post-pubertal	31	16.39	17.04	12.34	18.32
Male	Pubertal phase	Pre-pubertal	20	8.93	8.68	6.46	12.43
		Post-pubertal	28	16.7	17.08	13.45	18.8

Table 3-1. Healthy volunteers (n=99) summarised by pubertal phase, sex and age.

Ethnicity	n	%
Not reported	4	4.00%
Black African	5	5.10%
Black Caribbean	1	1.00%
East Asian	2	2.00%
Mixed Background	7	7.10%
Other	4	4.00%
South Asian	22	22.20%
White	54	54.50%

Table 3-2. Healthy volunteer ethnicity

All of the volunteers younger than 13 years old were recruited during routine dental or orthodontic surgery for caries, extraction or orthodontic procedures. All were reported to be well at the time of surgery. Three patients were on asthma and hayfever medication. None of the young people were on oral contraceptives and all were asked about current colds and flu like symptoms and reported being well at recruitment. Serum sex hormone levels were

assessed against self reported pubertal phases, and corresponded with expected normal levels in these groups (Fig. 3-1).

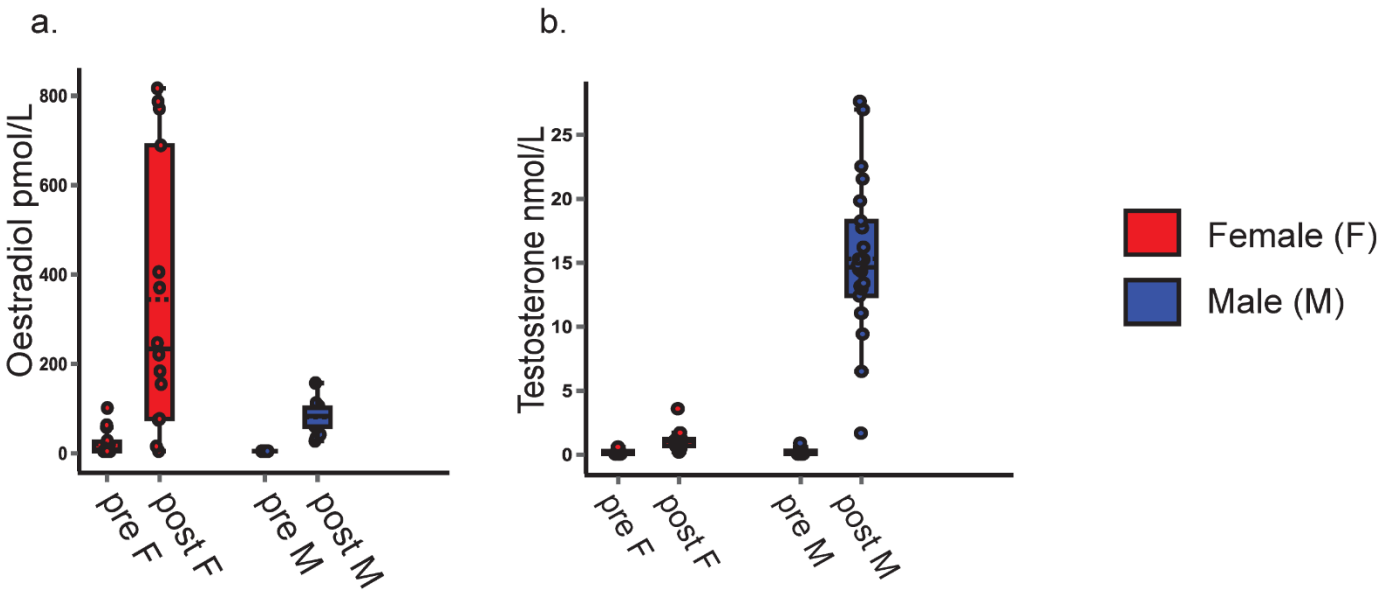


Figure 3-1. Pubertal phase self reporting associated with expected serum sex hormone concentrations. (pre=pre-puberty; post=post-puberty)

3.2. Transgender volunteers

Sixteen transgender males (Trans M-XX) and 7 transgender females (Trans F-XY) were recruited. Three of the transgender males were excluded as they had serum testosterone concentrations greater than 60nmol/L which is higher than upper limit of normal for an adult male. This may be due to the young people being recruited on the day of their testosterone injection, or due to young people intentionally using larger doses than recommended. In any case, these young volunteers were excluded as their data influenced the results of the investigations including serum testosterone concentration as a variable.

All of the transgender volunteers had completed the phase of therapy with pubertal blockers (GnRH agonists alone). All volunteers had received at least 3 months of therapy with either testosterone or oestradiol. It should be noted that trans female patients remain on GnRH agonists during their therapy with alternate sex hormone. The age of transgender volunteers is represented in Table 3-3 below.

		Age		
	n	Median	Min	Max
Trans Male (XX)	13	18.83	17.63	19.46
Trans Female (XY)	7	18.61	17.3	19.53

Table 3-3. Transgender volunteers by age

3.3. Turner's syndrome volunteers

Young women with TUS were recruited from the University College London Hospital (UCLH) endocrinology clinic. Young women were recruited regardless of genotype, if they displayed the clinical phenotype of TUS. As mentioned in the introduction, TUS manifests phenotypically when there is an insufficiency of the second X chromosome. The features and genotype of these young volunteers are represented below in Table 3-4.

Age	Therapy	Genetic diagnosis
15.3	Oestradiol	Turner variant, deletion of chromosome Xq21.3
19.6	Oestradiol and growth hormone	Turner mosaicism (45,X/46,X,idic(X)(p11.2)
11.4	Growth hormone	Turner syndrome 45,X
15.2	Oestradiol and growth hormone	Turner syndrome 45,X
18.2	Thyroxine and oestradiol	Turner 45,X
15.9	Growth hormone	Turner mosaic (45,X/46,XX)
15.8	Oestradiol and growth hormone	45,X Turner
16.7	Vitamin D	Turner mosaic 45,X/47,XXX
13.8	Growth hormone and vitamin D	Turner mosaic 47,XXX/45,X

Table 3-4. Features of young volunteers with Turner's syndrome.

3.4. Building a model to test the effect of X chromosome number vs serum sex hormone concentration.

As discussed in the introduction, it is unknown whether sex differences exist in the TLR7 mediated production of IFNα before sexual maturity. Serum sex hormone concentration and X chromosome number are inherently linked in healthy adults. Therefore, a population model was built, using the volunteers recruited above, in order to uncouple the traditional correlation between X chromosome number and serum sex hormone concentration. By preventing the inherent collinearity in these variables, a regression model was built that allowed for the measurement of the individual contributions of X chromosome number, serum oestradiol and serum testosterone concentrations to the variability in the outcome measures of interest. The premise behind this model is represented in Figure 3-2 which shows a full range of serum testosterone and oestradiol concentrations on the background of either one or two X chromosomes.

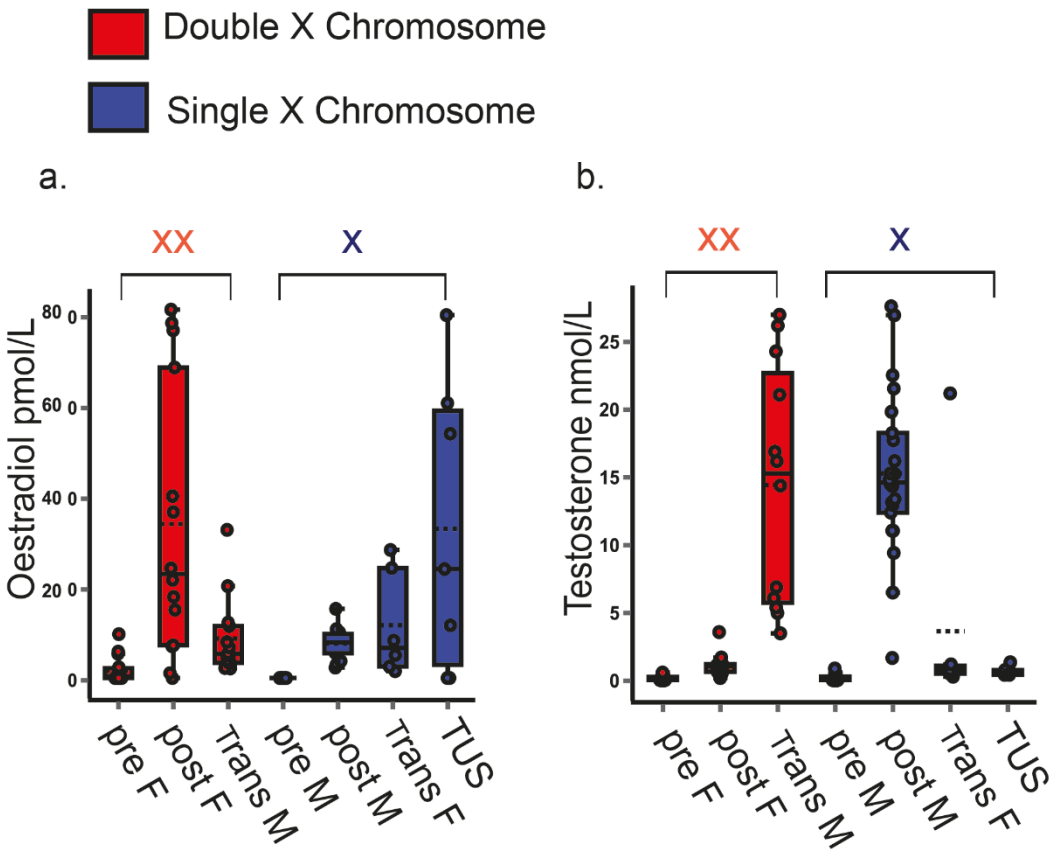


Figure 3-2. Population model. a-b. Serum hormone levels in pre- and post-pubertal healthy, transgender and TUS volunteers allowed for a full spectrum of hormone concentration upon the background of one or two X chromosomes (n=135). Pre F -Pre pubertal female; post F-post pubertal female; Trans M-Transgender Male (XX); Pre M-Prepubertal male; post M -post pubertal male; trans M- transgender female (XY); TUS-Turners syndrome

3.5. Volunteers with jSLE

Thirty-nine patients attending the UCLH adolescent rheumatology clinic with jSLE were recruited. These included 26 females and 13 males. Median age was 17.2 with a maximum of 20.7 and a minimum of 14.7. In order to minimise the effect of disease activity and exogenous steroid therapy on the IFN based assays, participants with a low disease activity (SLEDAI<5), on relatively low doses of steroid therapy were specifically recruited (Table 3-5). Only one female and one male had received a dose of intravenous methylprednisolone within 3 months of sampling. The ethnicity of one male was unknown. The ethnicities of the group are summarised in Table 3-6. In keeping with known trends, in the healthy volunteers above, 54% were white, whereas only 25% of the young volunteers with jSLE were white.

For the purposes of description, the therapies, organ involvement and antibody profiles of the volunteers with jSLE are represented in Tables 3-6,7,8. There was only 1 volunteer who was newly diagnosed and not on any therapy.

	Female (n=26)		Male (n=13)	
	Median	Range	Median	Range
Age	16.67	14.7-17.7	18.2	16.4-20.7
Average SLEDAI	2	0-12	2	0-24
CRP (mg/L)	0.60	0.06-10.7	1.00	0.6-18
WCC (x10^9/L)	4.97	1.0-10.0	4.97	2.75-7.00
C3 (g/L)	1.07	0.61-1.77	.93	0.33-1.30
C4 (g/L)	.19	0.06-0.43	.14	0-0.27
ESR (mm/hr.)	11	2.0-29.0	10	0-47
dsDNA (IU/ml)	26	0-687	62	0-2827
Median daily dose prednisone (mg)	3	0-15	0	0-15

Table 3-5. Characteristics of young volunteers with jSLE. SLEDAI-Systemic lupus erythematosus disease activity index, CRP-C reactive protein, WCC-White cell count, C3/4-complement 3 or 4, ESR-Erythrocyte sedimentation rate, dSDNA –anti-double stranded DNA antibody titre

		Sex		Total (%)
		Female	Male	
Ethnic groups	White	7	3	10 (25.6)
	South Asian	5	2	7 (17.9)
	Mixed	3	0	3 (7.7)
	Black	4	4	8 (20.5)
	East Asian	3	3	6 (15.4)
	Other	4	0	4 (10.3)
Total		26	12	38

Table 3-6. Ethnic groups of jSLE volunteers.

	FEMALE (26)		MALE (13)	
	n	%	n	%
Prednisone	15	57.69	5	38.46
Steroid pulse in last 3 months	1	3.85	1	7.69
Mycophenylate	10	38.46	4	30.77
Methotrexate	1	3.85	1	7.69
Azathioprine	11	42.31	5	38.46
Rituximab ever	7	26.92	2	15.38
Belimumab ever	1	3.85	1	7.69
Cyclophosphamide ever	3	11.54	1	7.69
Hydroxychloroquine	26	100.00	12	92.31

Table 3-7. Therapy at time of recruitment. (Belimumab, Rituximab and cyclophosphamide recorded if ever used)

	FEMALE (26)		MALE (13)	
	n	%	n	%
Skin	23	88.46	12	92.31
Haematological	12	46.15	6	46.15
Neurological	2	7.69	0	0.00
Renal	8	30.77	3	23.08
Joint	14	53.85	6	46.15
Cardiac	3	11.54	4	30.77

Table 3-8. Organ involvement ever recorded.

	FEMALE (26)		MALE (13)	
	n	%	n	%
Anti-RNP	5	19.23	5	38.46
Anti-Sm	5	19.23	4	30.77
Anti-La	3	11.54	2	15.38
Anti-Ro	12	46.15	4	30.77
Anti-phospholipid antibodies	1	3.85	1	7.69

Table 3-9. Antibodies present at time of recruitment. RNP-Ribonucleoprotein, Sm-Smith, antiphosphoplipid antibodies may include anti-lupus anticoagulant, anti-β2 glycoprotein or anti-cardiolipin.

3.6 Sample numbers

There were not always sufficient cell numbers obtained from each volunteer for all of the experiments. Although the sample numbers are represented in the graphs below in the results chapters, a summary is provided below of the data numbers for each data type in Table 3-10, which will serve as a reference for the results chapters below if necessary.

		Puberty	Ex vivo flow	R848 %pDC IFNα+	CpG %pDC IFNα+	R848 IFNα (pg./ml)	CpG IFNα (pg./ml)	Ex vivo GE	Post stim GE	Serum Oestradiol	Serum Testosterone
Healthy	F	Pre	19	20	18	18	15	14	9	16	19
		Post	37	31	28	27	24	10	7	14	20
	M	Pre	19	20	19	19	17	12	10	13	18
		Post	31	28	25	22	18	14	7	17	22
TUS	F	Post	9	9	9	8	6	9	6	7	7
Trans	M	Post	13	13	11	11	6	12	7	11	12
	F	Post	7	7	7	6	4	6	5	6	7
jSLE	F	Post	19	26	22	16	13	20	9	18	19
	M	Post	11	13	9	6	5	9	1	8	8

Table 3-10. Numbers of data available for each outcome measure. *GE= gene expression

Chapter 4: Plasmacytoid dendritic cell phenotype, sex and puberty.

It is unknown whether sex and pubertal phase associate with significant changes in the percentage, activation and tetherin protein expression of pDC and other cell types within PBMC in young people. It is also not known whether X chromosome number or sex hormone concentration associate with these changes.

4.1. Sex and pubertal differences in the percentage of PBMC subtypes.

Aim: To assess whether sex or pubertal differences exist in the percentage of pDC in PBMC in healthy young people.

Sub-aim: To investigate whether sex or pubertal differences exist in the percentages of other cell subtypes in healthy young people.

In healthy volunteers only, *ex vivo* flow cytometry was performed to assess for the percentage of CD14+ monocytes, CD19+ B cells, CD4+ T cells, CD8 + T cells, BDCA2+ pDC, CD11c+ conventional dendritic cells (cDC) and CD56+ NK cells within PBMC .

The association of sex and pubertal phase, with the variability of the percentage of each cell type in the PBMC compartment, was investigated with the following linear regression model:

$$\%Cell\ type = Intercept + B_1 * Sex + B_2 * Pubertal\ phase$$

Al the data represented by the above model for each variable tested in this chapter is summarised in table 4-1.As a regression model with two independent variables was being performed for 7 cell types (dependant variable), the significance value of each coefficient for sex (B_1) and puberty (B_2) was corrected for multiple testing by Benjamini Hochberg method with a false discovery rate of 0.20 (Table 4-2).

	Model: $y=intercept+B_1*Sex+B_2*Puberty$										
Investigation	Dependant variable	Model predictive value	Model significance	Independent Variables							
				Sex (F=1; M=0)				Puberty(Pre=0; Post=1)			
		Adjusted R^2	p	B_1	p	95% CI		B_2	p	95% CI	
% Cells in PBMC	%pDC	0.009	0.249	0.005	0.889	-0.064	0.073	-0.059	0.097	-0.129	0.011
	%CD4	0.058	0.024	4.074	0.01	1.003	7.145	1.279	0.418	-1.846	4.405
	%CD14	0.174	0.001	-1.127	0.152	-2.677	0.422	3.596	0.001	2.013	5.179
	%CD11c	0.06	0.043	-0.181	0.025	-0.339	-0.023	0.1	0.221	-0.061	0.262
APC CD86 expression	%pDC CD86	0.041	0.052	1.88	0.021	0.294	3.465	-0.671	0.413	-2.291	0.949
	%B cell CD86+	0.088	0.005	0.673	0.181	-0.3191	0.665	-1.586	0.003	-2.6	-0.572
	%CD14 CD86+	0.06	0.021	0.045	0.303	-0.041	0.132	-0.119	0.009	-0.208	-0.031
Tetherin expression	pDC tetherin	0.053	0.029	75.767	0.011	17.568	133.965	-27.304	0.364	-86.757	32.149
	B cell tetherin	0.039	0.059	54.156	0.023	6.229	84.082	13.238	0.51	-26.527	53.003

Table 4-1. Summary of linear regression models investigating the effect of sex and puberty on PBMC pheontypes.

Dependant and Independent variable	Original p value	Benjamini-Hochberg significance	Benjamini-Hochberg P-value
%CD14 monocytes puberty	0.001	**significant**	0.014
%CD4+ T cells sex	0.01	**significant**	0.07
%cDC sex	0.025	**significant**	0.107333333
% B cells puberty	0.053	**significant**	0.1855
%pDC puberty	0.097	**not significant**	0.38675
%CD14 monocytes sex	0.154	**not significant**	0.38675
% B cells sex	0.21	**not significant**	0.38675
%cDC puberty	0.221	**not significant**	0.38675
%CD4+ T cells puberty	0.428	**not significant**	0.665777778
%CD8+ T cells sex	0.567	**not significant**	0.775090909
%NK cell sex	0.609	**not significant**	0.775090909
%pDC sex	0.682	**not significant**	0.778
%CD8+ T cells puberty	0.74	**not significant**	0.778
%NK cell puberty	0.778	**not significant**	0.778

Table 4-2. Multiple regression for the association of cell type percentage in PBMC with sex and puberty corrected for a false discovery rate of 0.20 by Benjamini Hochberg method.
Highlighted values are significant after correction. (Benjamini Hochberg p values<0.2 considered significant)

4.1.1. The percentage of pDC in PBMC did not differ significantly between sexes or pubertal phases in healthy volunteers.

There were no significant differences in the percentage of pDC in PBMC between sexes or pubertal phases, after correcting for each other (Fig. 4-1a).

4.1.2. Sex and pubertal differences were seen in the percentage of CD4+ T cell, CD14+ monocyte and CD11c+ cDC subpopulations in healthy volunteers.

The percentage of CD4+ T cells was on average 4% higher in females (B_1=4.03; p=0.010; CI=0.990, 7.073) (Fig. 4-1b), with no significant association with pubertal phase. There were on average 0.18% more conventional CD14-/CD11c+ cDC in males (B_2=0.182; p=0.023; CI=0.025, 0.339) with no association with pubertal phase (Fig 4-1c). Post pubertal healthy volunteers had, on average 3.6% more CD14+ monocytes than pre-pubertal volunteers (B_2=3.61; p<0.001; CI=2.040, 5.179), with no significant association with sex (Fig. 4-1d). No significant sex or pubertal differences were seen in the percentage of CD19+ B cells, CD8+ T cells or CD56+ NK cells.

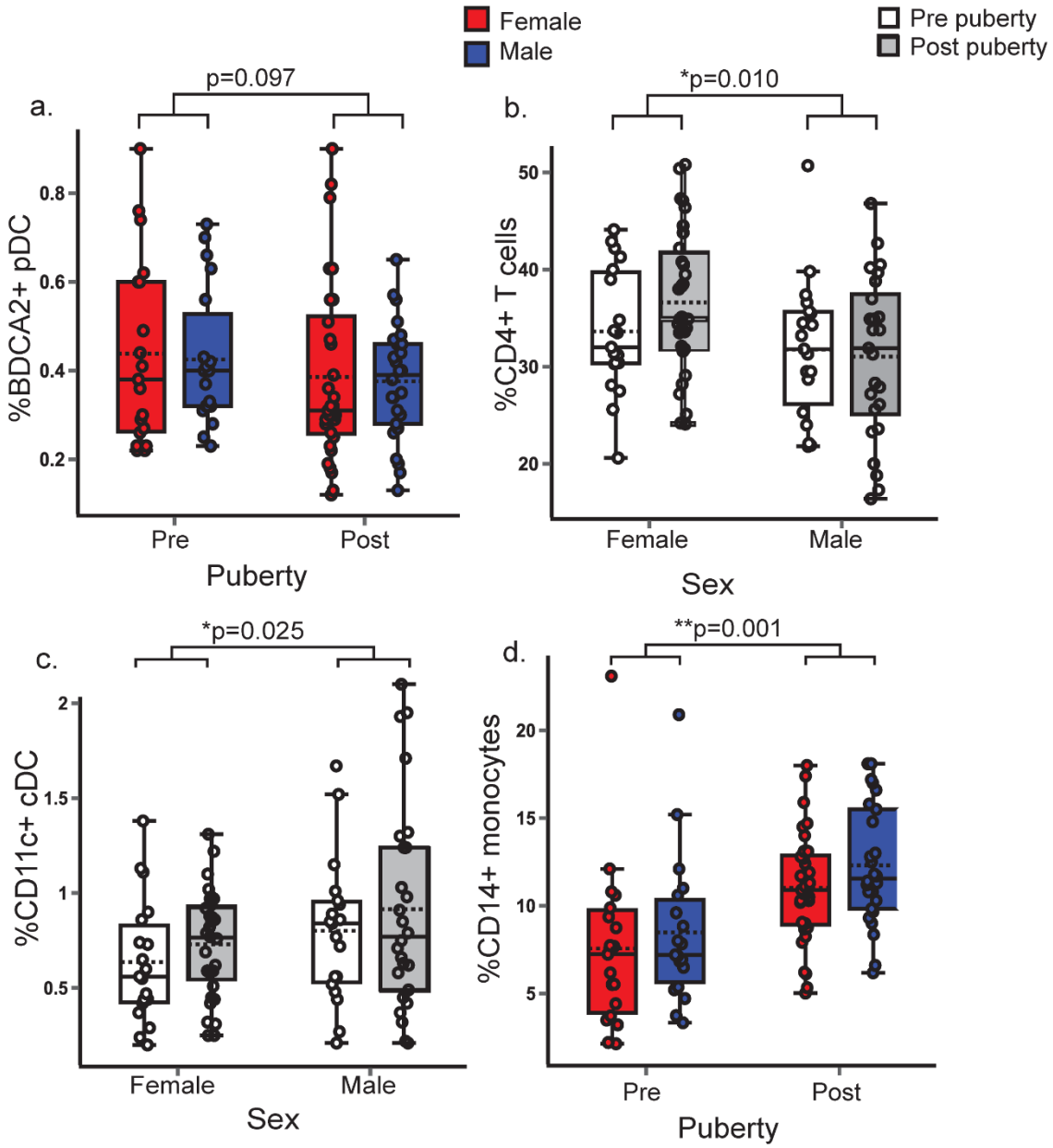


Figure 4-1. PBMC cell subtype percentage in healthy volunteers. Healthy volunteer PBMC were assessed by flow cytometry (n=106:pre F=19; post F=37; pre M =19; Post M=31). p values represent the significance of the coefficient the variable shown as estimated by linear regression after correcting for the other variable in the model (sex or puberty). a. %BDCA2+ pDC did not differ with pubertal phase (p=0.097). b. % CD4+ T cells was higher in females (p=0.010). c. %CD14-/CD11c+ cDC was higher in males (p=0.025) d. % CD14+ monocytes was higher in post-pubertal volunteers (p=0.001).

4.2. Sex and pubertal differences in pDC activation.

Aim: To investigate whether the expression of the activation marker CD86 on pDC differs significantly between sexes or pubertal phases in healthy young people.

Sub-aim: To investigate whether expression of CD86 on other antigen presenting cells (APC) differs significantly between sexes or pubertal phases in healthy young people.

CD86 is a T cell costimulatory molecule and marker of activation, expressed on APC(254). The surface expression of CD 86 was assessed by flow cytometry in healthy volunteers only .

4.2.1. pDC were significantly more activated in female volunteers than male, regardless of pubertal phase.

pDC were the only cell type which showed a significant sex difference in the surface expression of CD86. Females, on average, had 1.9% more pDC expressing CD86 than males (p=1.89; p=0.021; CI=0.307, 3.447) (Fig. 4-2a), with no significant association with pubertal phase.

4.2.2. Monocytes and B cells were significantly less activated in post pubertal volunteers, with no sex differences.

CD19+ B cells (B=-1.531; p=0.004; CI=-2.553, -0.510) (Fig. 4-2b) and CD14+ monocytes (B=-0.120; p=0.008; CI=-0.208, -0.033) (Fig. 4-2c) both expressed significantly less surface CD86 in post pubertal volunteers. There were no significant associations between CD19+ B cell or CD14+ monocyte CD86 expression and sex.

No significant sex or pubertal differences existed in the surface CD86 expression of CD11c+ cDC.

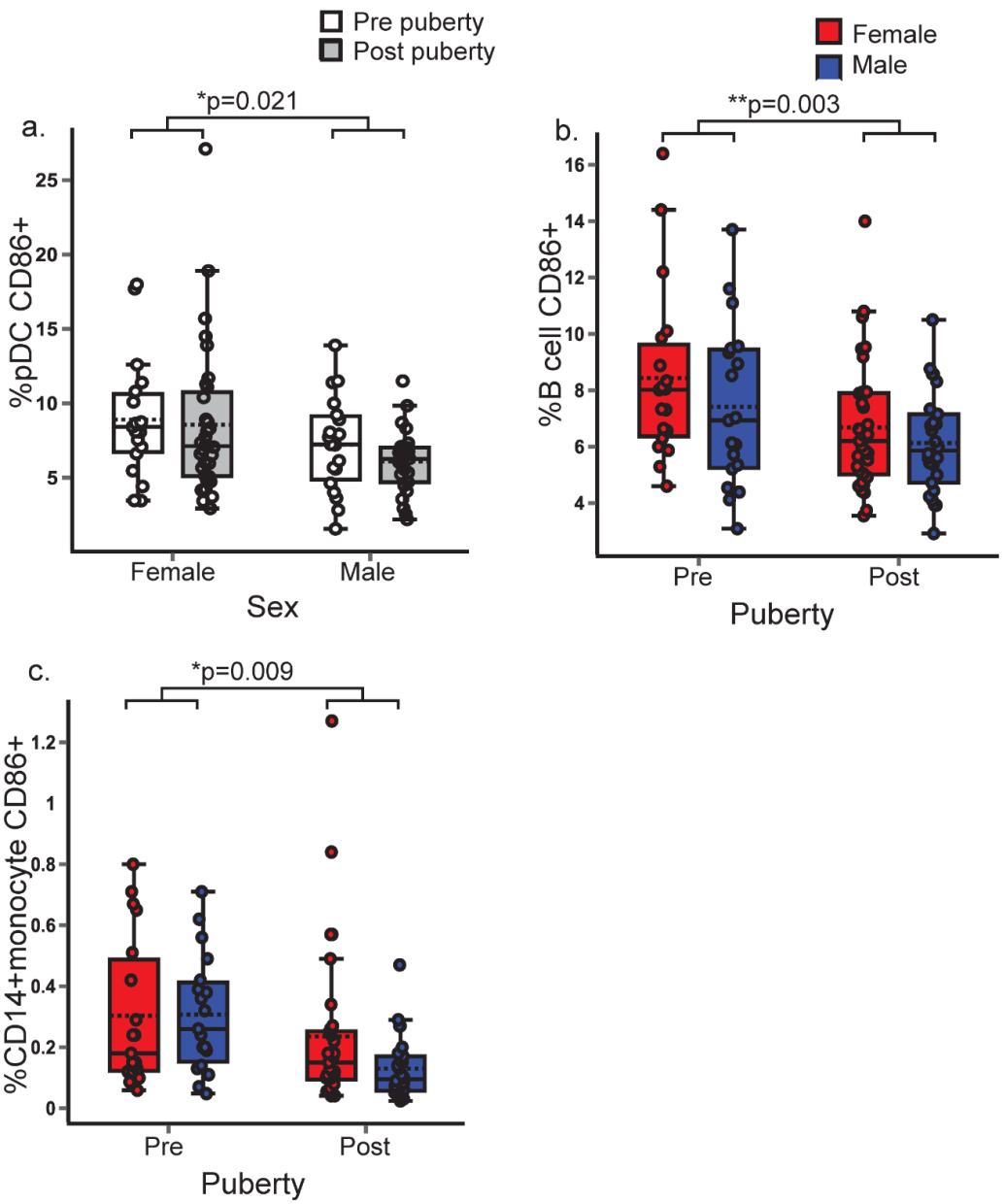


Figure 4-2. CD86 expression in pDC, B cells and monocytes in healthy volunteers. CD86 expression was measured on *ex-vivo* APC by flow cytometry and association with sex or pubertal phase was assessed by linear regression (n=106:pre F=19; post F=37; pre M =19; Post M=31). a. In females, a higher percentage of pDC expressed CD86 than in males (p=0.021), with no significant association with pubertal phase. b. - c. In post pubertal volunteers, there was a significantly lower percentage of B cells (p=0.003) and CD14+ monocytes (p=0.009) expressing surface CD86, with no significant association with sex. P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

4.3. X chromosome and sex hormone associations with pDC activation.

Aim: To investigate whether pDC CD86 expression associates with X chromosome number, serum oestradiol and testosterone concentration in healthy volunteers, transgender and TUS volunteers.

Sub-aim: To investigate whether CD86 expression on other APC associates with X chromosome number, serum oestradiol and testosterone concentration in healthy volunteers, transgender and TUS volunteers.

The following regression model was fitted:

$$\%\text{Cell type CD86+} = \text{Intercept} + B_1 * \text{X chromosome number} + B_2 * \text{Testosterone} + B_3 * \text{Oestradiol}$$

The data from this regression model investigating CD86 expression on APC and B cell and pDC tetherin expression (see later) is summarised in table 4-3 below.

	Model: y=intercept+B_1*X Chromosome number+B_2*Testosterone+B_3*Oestradiol															
		Model predictive value	Model significance	Independent Variables												
	Dependant variable	Adjusted R^2	p	X chromosome number (1=0;2=1)				Testosterone				Oestradiol				
				B_1	p	95% CI		B_2	p	95% CI		B_3	p	95% CI		
APC CD86 expression	%pDC CD86+	0.083	0.017	2.406	0.007	0.66	4.152	-0.59	0.05	-0.17	0.05	0.01	0.664	-0.01	0.01	
	%B cell CD86+	0.06	0.043	0.94	0.097	-0.18	2.056	-0.04	0.23	-0.11	0.03	-0.01	0.036	-0.01	0	
Tetherin expression	pDC tetherin	0.044	0.082	68.082	0.073	-6.62	142.783	-3.54	0.13	-8.11	1.04	-0.08	0.465	-0.30	0.14	
	B cell tetherin	0.031	0.132	45.874	0.04	2.25	89.498	-1.09	0.42	-3.76	1.58	-0.22	0.731	-0.15	0.11	

Table 4-3. Summary of linear regression models assessing for the effect of X chromosome number, serum testosterone and oestradiol on cell surface CD86 and tetherin expression .

4.3.1. pDC surface CD86 expression was higher if two X chromosomes were present.

After correcting for serum sex hormone, it was found that the presence of two X chromosomes associated with, on average, 2.4% more pDC expressing surface CD86 (B_1=2.406; p=0.007; CI=0.660, 4.152) There was no significant association with serum testosterone or oestradiol concentration (Fig. 4-3).

There was a non-significant trend towards a higher percentage of CD19+ B cells expressing CD86 in the presence of two X chromosomes (B_1=0.940, p=0.097; CI=-0.175, 2.056). CD14+ monocyte CD86 expression did not differ significantly with X chromosome number.

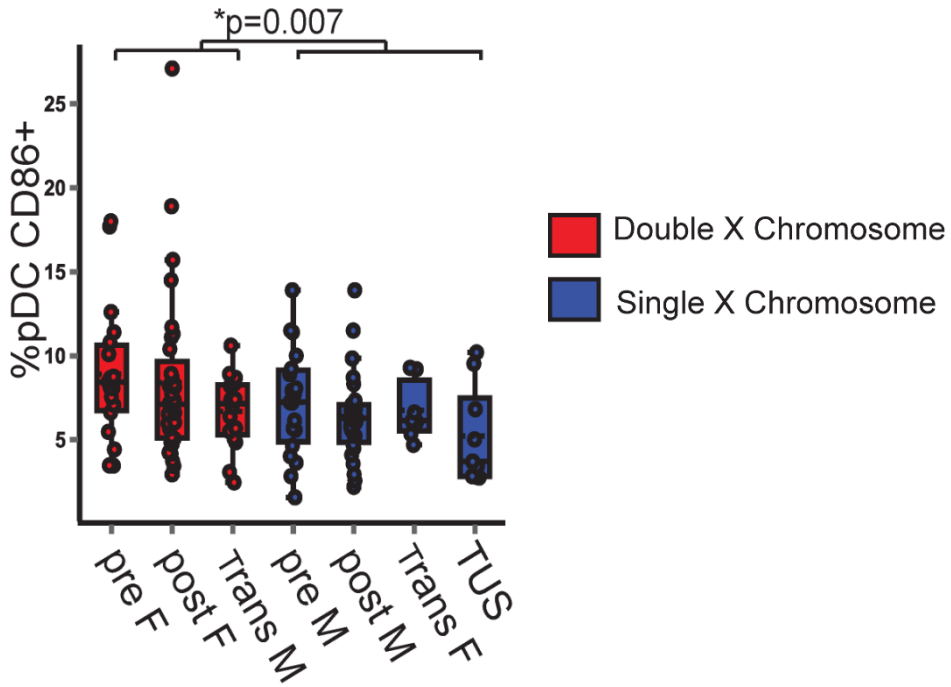


Figure 4-3. pDC CD86 expression was higher if two X chromosome were present. a. The percentage of pDC expressing CD86 in healthy, transgender and TUS volunteers was measured. a. A linear regression model revealed that the presence of two X chromosomes associated with a higher pDC surface expression of CD86 (p=0.007) with no significant association with serum testosterone or oestradiol concentration. (n=135; pre F=19; post F=37; Trans M=13; pre M=19; post M=31; Trans F=7; TUS=9). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

4.4. Sex and pubertal differences in the surface expression of an ISG product (tetherin protein) in pDC and B cells.

Aim: To investigate whether pDC tetherin expression is significantly different between sexes or pubertal phases in healthy volunteers.

Sub-aim: To investigate whether B cell tetherin expression is significantly different between sexes or pubertal phases in healthy volunteers.

Tetherin is a type 1 IFN inducible antiviral membrane protein constitutively expressed by pDC and B cells (255, 256). There is evidence that tetherin may be used as a proxy to ISG measurement(257). Within this context, we have used tetherin as an example of an ISG product/surface protein to investigate whether differences exist across sex and puberty.

Tetherin expression was measured by flow cytometry. Linear regression models were fitted to evaluate for associations of sex and pubertal phase with B cell or pDC tetherin median fluorescent intensity (MFI) as in 4.1.

4.4.1. pDC and B cells from females expressed more tetherin than males, regardless of puberty.

In healthy females, *ex vivo* pDC had a significantly higher tetherin MFI than in males (B=79.108; p=0.011; CI=21.021, 137.187), with no significant association with pubertal phase (Fig. 4-4a).

In healthy females, *ex vivo* B cells had a significantly higher tetherin MFI than males (B=45.265; p=0.023; CI=6.723, 83.808), with no significant association with pubertal phase (Fig.4-4b).

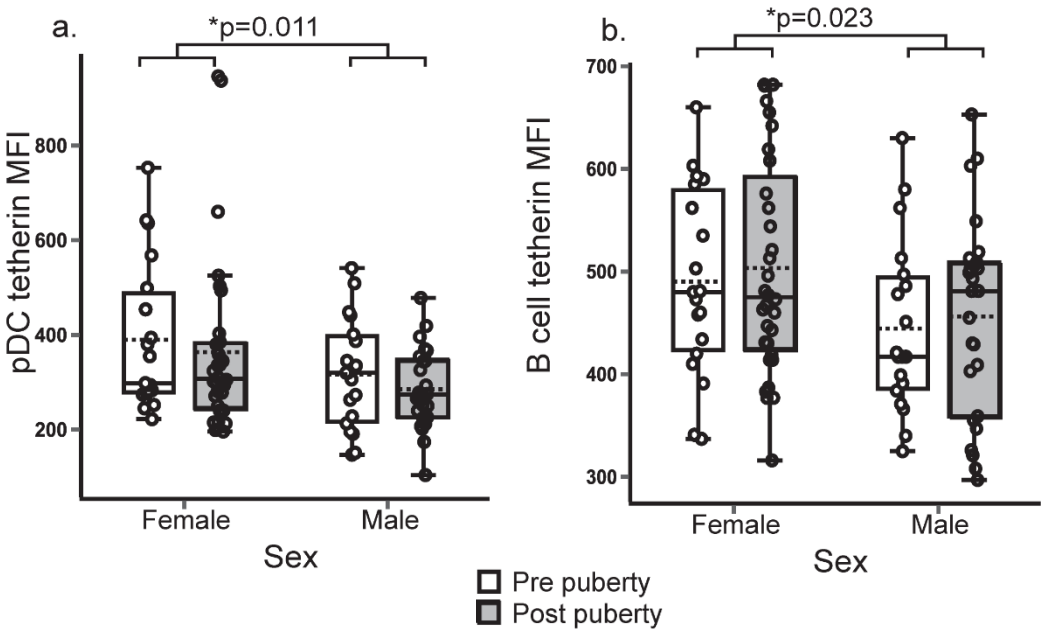


Figure 4-4. pDC and B cell tetherin expression in healthy volunteers. In healthy females, a. pDC (p=0.011) and b. B cells (p=0.023) had a significantly higher tetherin MFI, when compared to males, after controlling for pubertal phase. (n=106:pre F=19; post F=37; pre M =19; Post M=31). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

4.5. X chromosome and sex hormone associations with pDC and B cell expression of tetherin.

Aim: To investigate whether pDC tetherin expression associates with X chromosome number, serum oestradiol and testosterone concentration in healthy, transgender and TUS volunteers.

Sub-Aim: To investigate whether B cell tetherin expression associates with X chromosome number, serum oestradiol and testosterone concentration in healthy, transgender and TUS volunteers.

Linear regression was used as in 4.3. to analyse for associations with X chromosome number, serum oestradiol and testosterone concentration. See Table 4-3.

4.5.1. There was a non-significant trend towards an increased expression of tetherin in pDC if two X chromosomes were present.

The presence of two X chromosomes associated with a higher pDC tetherin MFI (B=68.082; p=0.073; CI=-6.618, 142.783) although this did not reach statistical significance, with no significant association with serum testosterone or oestradiol (Fig. 4-5a).

4.5.2. B cells expressed significantly more tetherin if two X chromosomes were present

B cell tetherin MFI was significantly higher in the presence of two X chromosomes (B=45.874; p=0.040; CI=2.249, 89.498) with no significant association with serum oestradiol or testosterone (Fig. 4-5b).

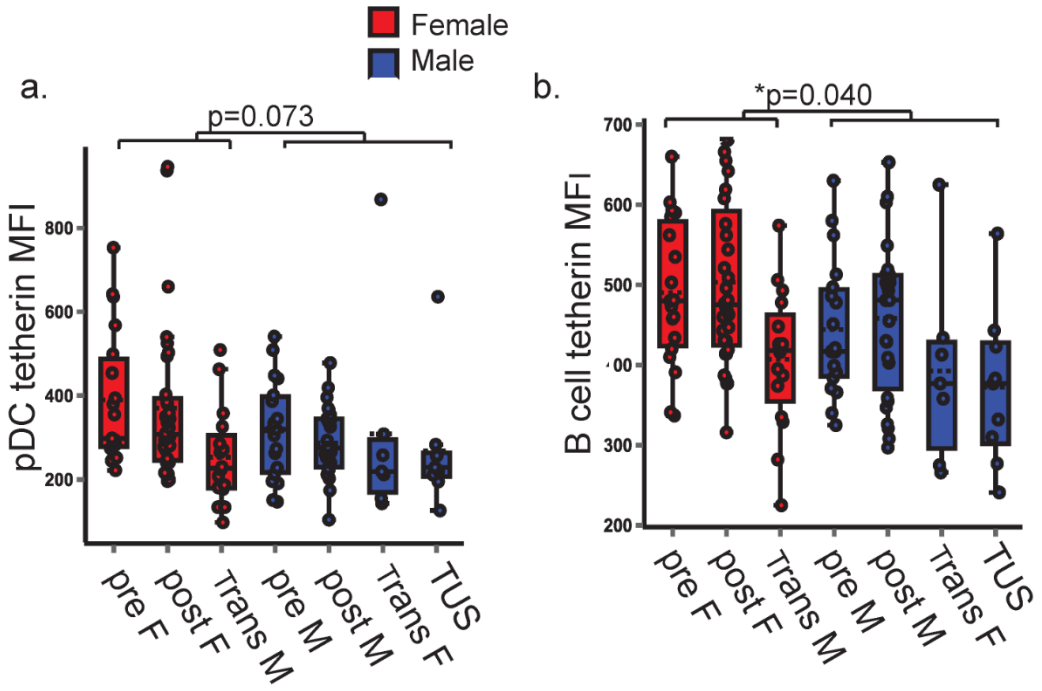


Figure 4-5. Tetherin expression in pDC and B cells associates with X chromosome number.a. After correcting for serum sex hormone concentration, the presence of two X chromosomes tended towards a higher pDC tetherin expression (p=0.073). b. B cell tetherin expression was higher in the presence of two X chromosomes (p=0.040). (n=135; pre F=19; post F=37; Trans M=13; pre M=19; post M=31; Trans F=7; TUS=9). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

.

4.6. Differences in pDC percentage, activation and ISG product expression between healthy volunteers and those with jSLE.

Aim: To investigate whether significant differences exist in the relative percentage, CD86 and tetherin expression of pDC between healthy volunteers and those with jSLE.

Sub-Aim: To investigate whether significant differences exist in the relative percentage, CD86 and tetherin expression of other immune cell types between healthy volunteers and those with jSLE.

pDC and other PBMC type parameters from patients with jSLE were compared to age and sex matched healthy volunteers by t-test or Mann-Whitney U test according to the distribution of the parameter. Five of the volunteers with jSLE had a B cell percentage of less than 2% as they had received rituximab therapy. The analysis was performed with and without these individuals for sensitivity analysis. Even though the inclusion of these individuals did not alter which cell types showed a significant difference between jSLE and healthy volunteers, they

were found to influence the relative percentage of other cell types, and therefore the estimated differences between the two groups. Therefore, it was decided to exclude them from the analysis of cell subtype percentage represented below (Fig. 4-8).

4.6.1. The percentage of pDC in PBMC was significantly reduced in jSLE

When compared by t-test, the percentage of BDCA2+ pDC in PBMC from patients with jSLE was significantly reduced when compared to healthy volunteers (p=0.01) (Fig. 4-6a) (Table 4-4).

4.6.2. The percentages of CD4+ T cells, CD8+ T cells, and CD11c+ cDC were significantly different in jSLE as compared to healthy volunteers.

The percentages of the other cell types within the PBMC compartment were analysed by t-test to assess for differences between healthy volunteers and those with jSLE. As there were multiple t-tests performed simultaneously, the results were checked for multiple testing using the Benjamini Hochberg method at a false discovery rate of 0.2 (Table 4-4).

The data are summarised in Table 4-4. Of note, there were on average 5.5% more CD4+ T cells in jSLE as compared to healthy volunteers (p=0.01) (Fig 4-6b). In the healthy volunteers previously analysed in 4.1.2., females had a higher percentage of CD4+ T cells than males. In order to confirm that the difference in the percentage of CD4 cells that existed between healthy volunteers and those with jSLE was not due to this previously described sex difference, a linear regression model was built including sex as a variable with disease status (Fig. 4-6c). In this model, participants with jSLE continued to have an increased percentage of CD4+ T cells regardless of sex (B=5.110; p=0.013; CI=1.115, 9.105) and females continued to have a higher percentage of CD4+ T cells regardless of disease status (B=4.88; p=0.009; CI=1.230, -8.53) (Fig. 4-6c).

In addition, the percentage of CD8+ T cells was significantly higher in jSLE (p=0.022) (Fig. 4-6d), while the percentage of CD11c+ cDC was significantly lower in jSLE (p=0.049) (Fig 4-6e) when compared to healthy volunteers. There were no significant differences in the percentage of CD19+ B cells (Fig 4-6f), NK cells or CD14+ monocytes within PBMC between jSLE and healthy volunteers. No other cell type showed a significant sex difference when analysed by linear regression, including patients with jSLE.

Cell subtype	p-value (t-test)	Significance after multiple testing	Benjamini Hochberg significance value	Mean Difference	95% Confidence Interval of the Difference	
%pDC	0.010	significant	0.035	0.101521	0.025144	0.177898
%CD4+ T cell	0.010	significant	0.035	5.4663	1.326	9.606
CD8+ T cell	0.022	significant	0.051333333	4.1937	0.623	7.763
%CD11c+	0.049	significant	0.08575	-0.19681	-0.00088	-0.39275
%NK cell	0.081	significant	0.1134	0.101297	-0.01271	0.215303
%CD14+ monocyte	0.854	not significant	0.93	0.17345	-1.69028	2.03718
% B cells	0.930	not significant	0.93	0.08106	-1.73989	1.902

Table 4-4. Differences in cell subtype percentage between healthy volunteers and those with jSLE. Cell type percentages were compared and corrected for multiple testing using Benjamini Hochberg at an FDR of 0.2. Highlighted values are significant. The mean difference and confidence intervals of the mean difference are shown.

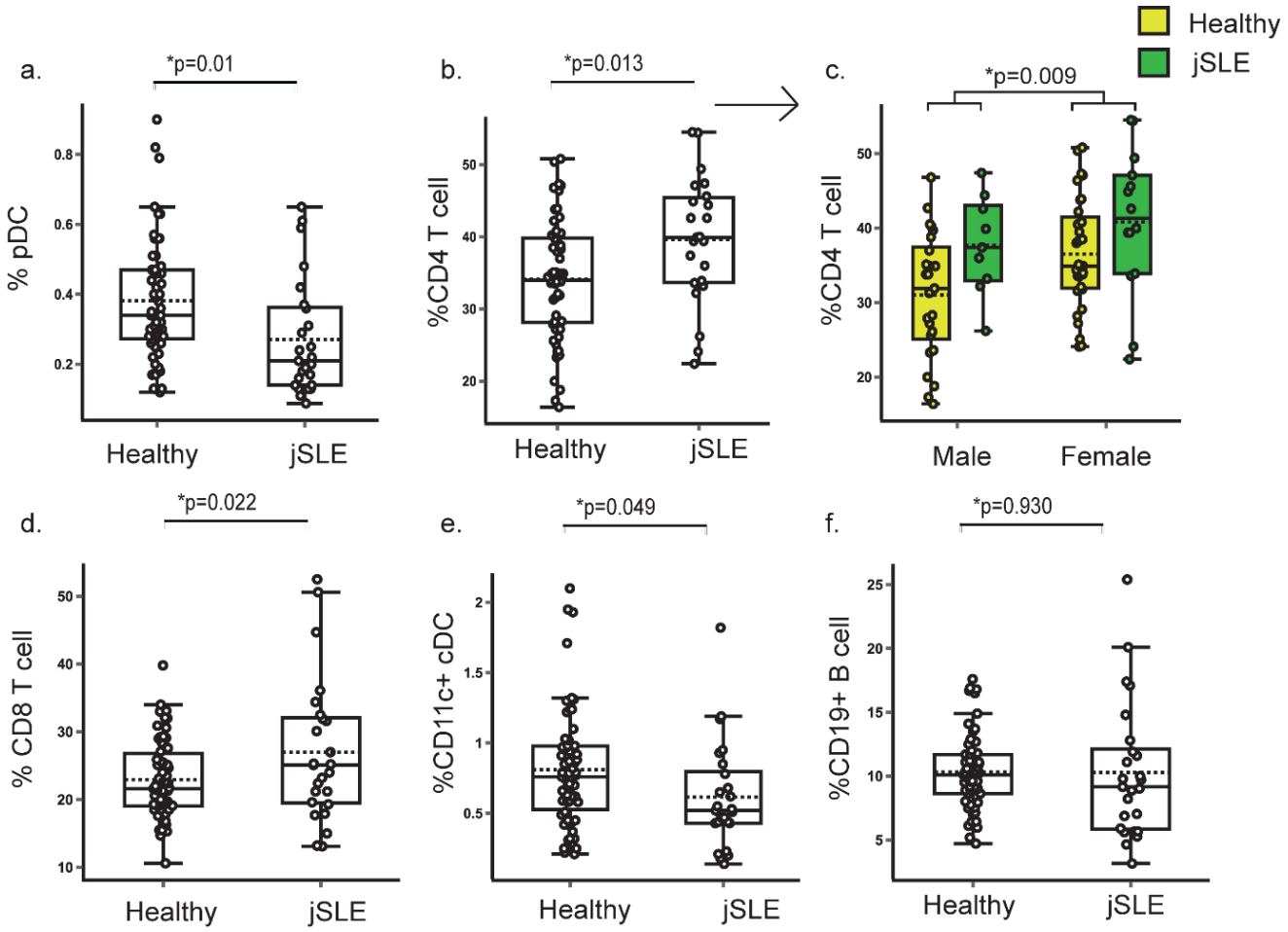


Figure 4-6. Differences in cell type percentage between healthy volunteers and those with jSLE

a. When analysed by t-test, and corrected for multiple testing, volunteers with jSLE (n=30-F-19, M-11) had a significantly lower percent of pDC in PBMC compared to post pubertal healthy volunteers (n=68, F-37, M-31). b. There was a significantly higher percent of CD4+ T cells in jSLE (p=0.01) when analysed by t-test. c. Upon correcting for disease (healthy vs jSLE) by linear regression, females still had a higher percentage of CD4+ T cells overall (p=0.009). d.CD8+ T cells (p=0.022), and e. CD11c+ cDC (p=0.049) showed significant differences between jSLE and healthy volunteers. There were no significant differences seen in the percentage of f. CD19+ B cells (p=0.930), CD14+ monocytes or CD56+ NK cells (not shown).

4.6.3. pDC were not significantly more activated in volunteers with jSLE, as compared to healthy volunteers.

There was a trend towards more pDC expressing CD86 in volunteers with jSLE that did not reach statistical significance (p=0.078) (Fig.4-7a), when correcting for sex, the trend remained (p=0.098).

4.6.4. CD14+ monocytes were significantly more activated in volunteers with jSLE.

CD14+ monocytes in volunteers with jSLE expressed significantly more CD86 when analysed by t-test (p=0.009). There was no significant difference in B cell CD86 expression between healthy volunteers and those with jSLE (p=0.476) (Fig. 4-7b, c).

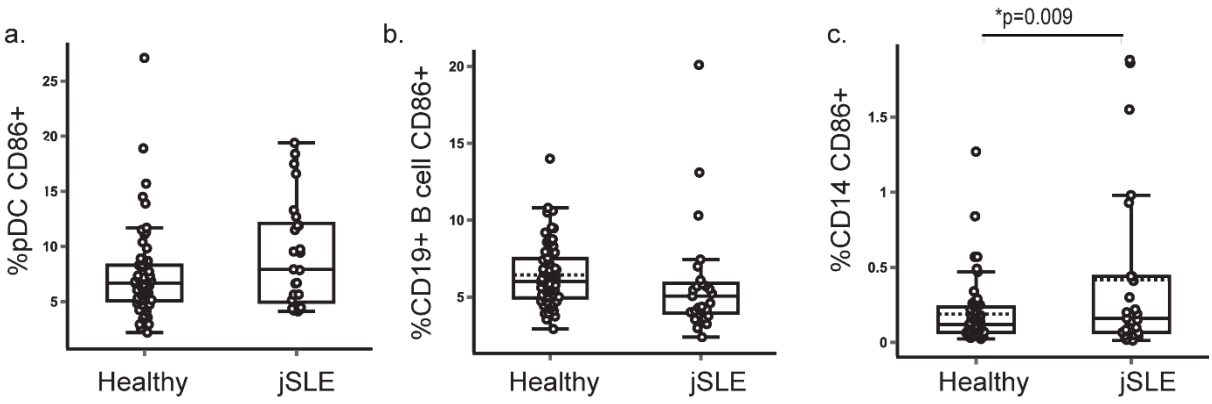


Figure 4-7. Differences in pDC, B cell and CD14+ monocyte activation between healthy volunteers and those with jSLE. a-b. When analysed by t-test, there was no difference in pDC or B cell CD86 expression between jSLE and healthy volunteers. c. The percentage of CD14+ monocytes expressing CD86 was significantly higher in jSLE than healthy volunteers (p=0.009) (n=98- SLE-30, healthy-68).

4.6.5. Tetherin expression on pDC and B cells was significantly higher in volunteers with jSLE.

Both pDC (p=0.001) and B cells (p=0.001) from patients with jSLE displayed an increased expression of surface tetherin compared to healthy young people when analysed by t-test (Fig. 4-8a; c). In healthy volunteers, pDC from females tended to express more tetherin (p=0.05), whereas this sex difference was lost in young people with jSLE (p=0.623) (t-tests) (Fig. 4-8b). B cell tetherin trended towards higher expression in healthy females (p=0.09) whereas this trend was not seen in patients with lupus (p=0.799) (Fig 4-8d).

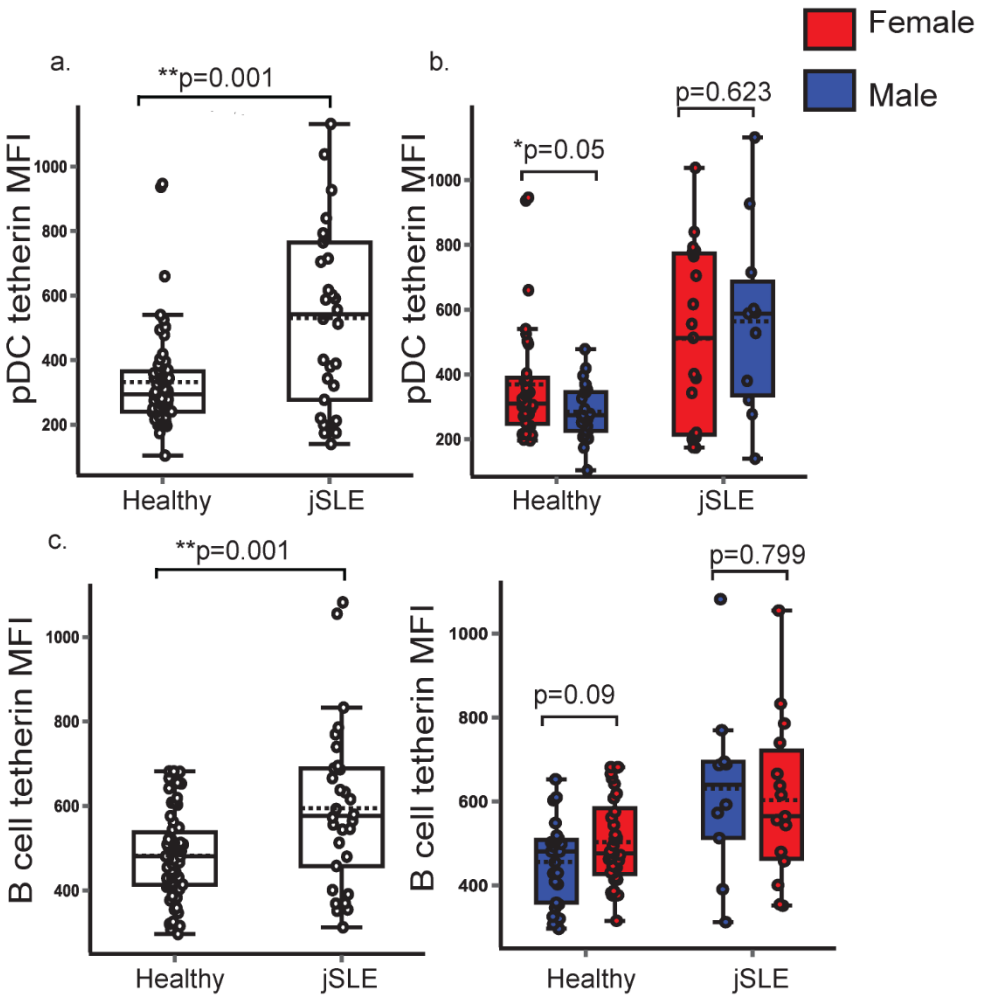


Figure 4-8. Differences in expression of the ISG surface protein, tetherin between healthy volunteers and those with jSLE. a. Tetherin expression was significantly higher in pDC (p=0.001) from volunteers with jSLE as compared to healthy volunteers when analysed by t-test. b. The sex difference in pDC tetherin expression was lost in jSLE. c. Tetherin expression was significantly higher in B cells (p=0.001) from volunteers with jSLE as compared to healthy volunteers. D. There was a trend towards a higher expression of tetherin in female B cells (p=0.09) which was not seen in SLE (p=0.799)(n=98-healthy=68, M-31, F=37, jSLE=30, M=11, F=19)

4.7. Discussion

In this chapter, it has been shown that, although the percent of pDC does not differ between sexes, pDC in females are significantly more activated and express significantly more of the ISG encoded surface protein, tetherin, than males. In addition, it has been shown that pDC activation and tetherin expression are related to X chromosome number, and not serum concentrations of oestradiol or testosterone.

It has previously been shown that the percentage of pDC in lymphocytes did not differ between healthy adult men and women (16, 120). In the data presented here, full blood counts were not available in healthy volunteers in order to confirm whether the absolute number of pDC per millilitre of blood was different between sexes, but the data agreed with the studies above in adults, in that the percentage of pDC in PBMC was not different between sexes. Previous data has shown that pDC percentage in PBMC may decline in the elderly(258). In these data, there was no significant difference in pDC percentage between pre and post pubertal volunteers. It is therefore proposed that if differences between sexes or pubertal phases exist in type 1 IFN production, they are most likely not due to differences in the relative quantity of pDC.

As has been described before(189), there was a decrease in the percentage of BDCA2+ pDC in volunteers with jSLE. It has been previously postulated that the decrease in pDC in peripheral blood in jSLE may be due to sequestration of pDC into peripheral sites of inflammation (35), but this has not been proven. In addition, BDCA2 is expressed highly on immature pDC and is downregulated upon TLR ligation of pDC(34). It is therefore possible that there is a decrease in the relative percentage of pDC expressing BDCA2, not due to a decrease in numbers of pDC, but rather due to a downregulation of BDCA2 in response to increased TLR ligation, or peripheral recruitment in jSLE.

Interestingly, females had a significantly higher percentage of CD4+ T cells than males. This has been described before in healthy adults and children (54-56) , as well as new-borns, children and adults in the context of HIV infection (57-59). In these data, this sex difference persisted even in patients with jSLE. The observation that females have a higher percentage of CD4+ T cells in healthy children, and those with jSLE or HIV infection suggests that this is an inherent and persistent difference. HIV and jSLE both have a type 1 IFN phenotype. It may be expected, if the sex difference in CD4+ T cell percentage was related to type 1 IFN, that it may be abolished in these diseases with an upregulated type 1 IFN. The fact that the sex difference persists despite a high background IFN in both sexes, implies that the difference in CD4+ T cell percentage is not related to the type 1 IFN pathway.

To the best of our knowledge, it has not been previously shown that sex differences exist in the activation of pDC *ex vivo* between sexes. In these data, in females, more pDC expressed CD86 than in males, regardless of pubertal phase. In addition, when the model was extended to include transgender and TUS volunteers, an association was seen with X chromosome number and not serum sex hormone concentration. These data imply that pDC are inherently,

chromosomally, more activated in females, as compared to males. pDC were the only APC type to show a significant sex difference in activation as measured by CD86.

A trend towards higher activation of pDC was seen in volunteers with jSLE when compared to healthy volunteers. The same caveat must be applied as above, in that pDC in jSLE may downregulate BDCA2 upon activation, or recruit peripherally, so the pDC that are being assessed here may not fully represent the entire quota of mature pDC in jSLE. In addition, the patients recruited with jSLE were specifically chosen with low disease activity. Therefore, the fact that a borderline insignificant trend towards higher activation of pDC is seen in jSLE should be noted.

Ex vivo tetherin expression was measured on B cells and pDC as a proxy marker for background type 1 IFN presence. Interestingly, pDC and B cells in healthy females expressed significantly more tetherin than males, regardless of pubertal phase. This too associated with X chromosome number and not serum sex hormone. This sex difference in tetherin expression has not previously been described. It allows for an intriguing hypothesis that females may have a higher expression of IFN inducible or 'anti-viral' proteins which may help to explain described differences in responses to vaccination or viral infection (17, 43, 45). It can be reasoned that the price that females pay for a higher anti-viral or type 1 IFN background, may be an increased susceptibility to autoimmune disease like jSLE.

Indeed, in jSLE, as would be expected of a disease with an abundance of type 1 IFN, pDC and B cell tetherin expression was significantly increased when compared to healthy volunteers. Intriguingly, the sex difference in pDC tetherin expression that was present in healthy volunteers was lost in volunteers with jSLE. This implies that although females have a higher propensity towards jSLE, with a higher background IFN phenotype, in males who develop the disease, this sex difference may be overwhelmed by the general increase in type 1 IFN.

4.8. Conclusion

In healthy females, pDC were more activated and expressed more of the anti-viral or type I IFN inducible surface protein, tetherin than males. This associated with X chromosome number and not sex hormone. In jSLE, there was an increase in the expression of tetherin on pDC and B cells, with a loss of the sex difference that was seen in healthy volunteers. In addition, pDC and B cells trended towards higher activation in jSLE.

After finding these inherent differences in pDC in the resting state, it was next investigated whether sex or pubertal differences existed in the pDC production of IFN and other cytokines in young people.

Chapter 5: Plasmacytoid dendritic cell function, sex and puberty

After it was shown in chapter 4 that *ex vivo* female pDC were more activated and trended towards expressing more tetherin when compared to males, it was investigated whether sex and pubertal differences existed in the ability of TLR7 or TLR9 activated pDC to produce IFNα.

5.1. Sex and pubertal differences in TLR mediated production of IFNα in healthy volunteers.

Aim: To investigate whether sex and pubertal differences exist in the percentage of pDC producing IFNα after R848 or CpG stimulation in healthy young volunteers.

Sub Aim: To investigate whether sex and pubertal differences exist in the amount of IFNα, IFNβ and TNFα produced by PBMC after R848 or CpG stimulation in healthy young volunteers.

5.1.2. Culture and flow cytometry

The percentage of IFNα producing pDC was measured in PBMC after stimulation with R848, CpG or no stimulation. An early ELISA experiment revealed that significantly more IFNα was produced by PBMC if they were stimulated with CpG as compared to R848 (Fig. 5-1). As pDC IFNα production after R848 stimulation was the primary outcome of interest, a dose titration was performed using 4 healthy volunteers, and it was confirmed that 1ug/ml was an adequate stimulation dose for R848 (Fig. 5-2).

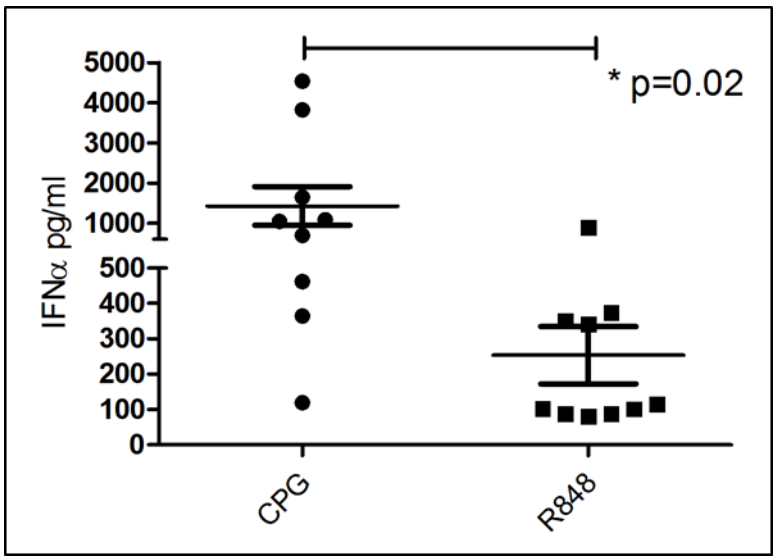


Figure 5-1. Preliminary ELISA data. Cells were cultured with CpG ODN 2216 1µM or R848 1µg/ml for 20 hours without BFA. Supernatant was analyzed by ELISA and it was shown that there was significantly more IFNα released after CpG stimulation.

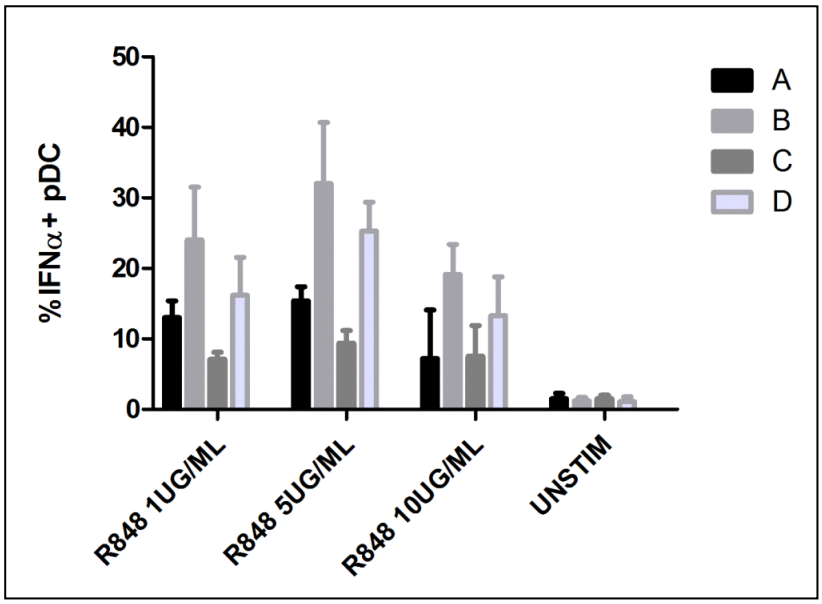


Figure 5-2. R848 dose titration. PBMC from 4 healthy adult donors (A, B, C, D) were stimulated for 20 hours with R848 at doses of 1µg/ml, 5µg/ml and 10µg/ml and the percentage of pDCs expressing IFNα was assessed by flow cytometry. It was found that 1µg/ml was an adequate dose for the assessment of IFNα production.

5.1.3. In healthy young people, there were no differences in background pDC IFNα production between sexes or pubertal phases.

It was first investigated whether any background sex or pubertal differences existed in the percentage of unstimulated pDC producing IFNα in healthy participants. The following linear regression model was fitted:

$$\%pDC\ IFN\alpha+=Intercept+B_1*Sex+B_2*Pubertal\ Phase$$

It was first established that there were no associations between the percentage of pDC producing IFNα and sex (B_1= -0.060; p=0.849; CI=-0.682, 0.562) or pubertal phase (B_2= -0.217; p=0.501; CI=-0.857, 0.423) if cells were not stimulated (Fig. 5-3).

In addition, it was confirmed that no sex or pubertal differences existed in the amount of IFNα, IFNβ or TNFα produced in supernatant by PBMC without stimulation (not shown).

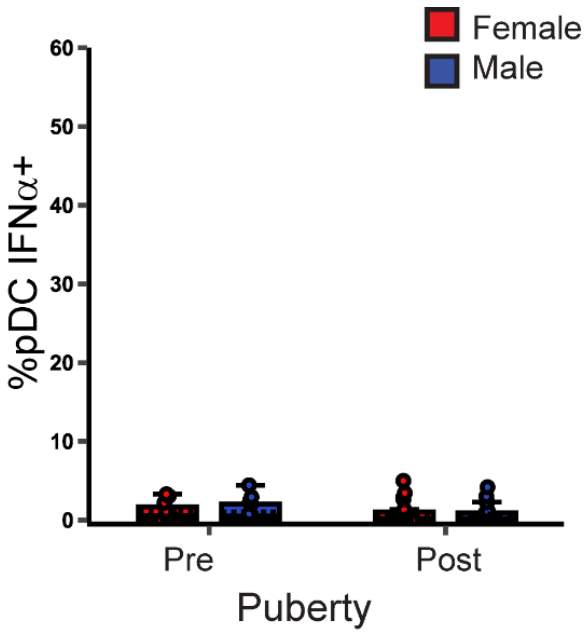


Fig. 5-3. Baseline pDC IFNα production did not differ between sexes or pubertal phases in healthy volunteers. No significant sex or pubertal differences existed in the percentage of pDC producing IFNα if cells were not stimulated.

5.1.4. After TLR7 stimulation, a higher percentage of pDC produced IFNα in healthy female and post pubertal volunteers.

PBMC from healthy, pre and post pubertal volunteers were stimulated with R848, and the percentage of pDC producing IFNα was measured by flow cytometry. The same linear regression analysis was performed as above in 5.1.3. The results are presented in Table 5-1 below.

The overall model was significant (p=0.004), and altogether, sex and pubertal phase predicted 9.2% of the variability in the percentage of pDC producing IFNα after R848 stimulation. Females had, on average, 8.3% more pDC producing IFNα after R848 stimulation than males after controlling for pubertal phase (p=0.008) (Fig. 5-4a). In addition, post pubertal volunteers had on average 6.5% more pDC producing IFNα after R848 stimulation after controlling for sex (p=0.039) (Fig. 5-4b).

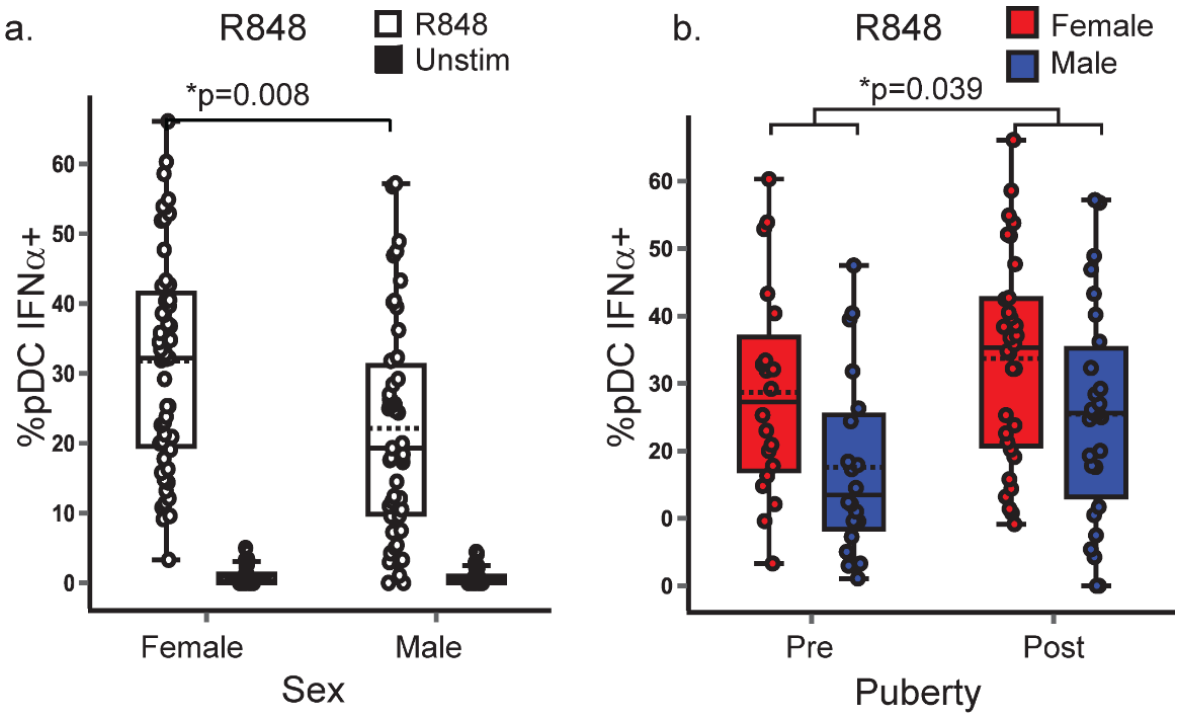


Figure 5-4. The percentage of pDC producing IFNα after R848 stimulation is higher in females and after puberty in healthy participants. After stimulation with R848, the percentage of pDC producing IFNα was measured and analysed by linear regression (n=99, Pre F=20, Pre M=20, Post F=31, Post M=28). a. There was a significantly higher percentage of pDC producing IFNα in female (p=0.008) and b. post pubertal volunteers (p=0.039). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

Dependant variable: Percentage pDC producing IFNα after R848 stimulation. Adjusted r^2=0.092; Model significance p=0.004				
Variables	B coefficient (B)	p	95.0% Confidence Interval for B	
(Constant)	27.308	0.000	21.607	33.009
Sex (F=0; M=1)	-8.334	0.008	-14.454	-2.214
Pubertal phase (pre=0; post=1)	6.569	0.039	0.336	12.802

Table 5-1. Regression model predicting the percentage of pDC producing IFNα after R848 stimulation.

5.1.5. After TLR7 stimulation, there was a trend towards a higher production of IFNα by PBMC in females and post pubertal healthy volunteers.

To confirm the findings in 5.1.4., PBMC from healthy volunteers were stimulated with R848 and total IFNα, IFNβ and TNFα in supernatant was measured by Luminex assay. There were slightly fewer samples with data (n=86) in this analysis as not all volunteers had sufficient cell numbers for all analyses. The same linear regression model was applied as in 5.1.3. As the concentration of cytokine data was skewed, the data was transformed using the natural log, in order to analyse by linear regression and maintain residuals with a normal distribution. The coefficients reported below are all reported as the exponential of the coefficient (Exp B) and are, accordingly, multiplicative in nature.

Overall, sex and puberty predicted 7.4% of the variability in the amount of IFNα produced by PBMC. The overall model was significant (r^2=0.074; p=0.043). After R848 stimulation, female PBMC produced 49% more IFNα than males (ExpB=0.49; p=0.074; CI=0.22-2.01) (Fig. 5-5a) and PBMC in post pubertal volunteers produced almost double the amount of IFNα than the pre-pubertal group (ExpB=1.94; p=0.081; CI=0.89-4.25) (Fig. 5-5b). These coefficients did not reach significance as observed in the pDC specific experiment in 5.1.4.

No significant differences were seen in the production of IFNβ or TNFα in supernatant after R848 stimulation.

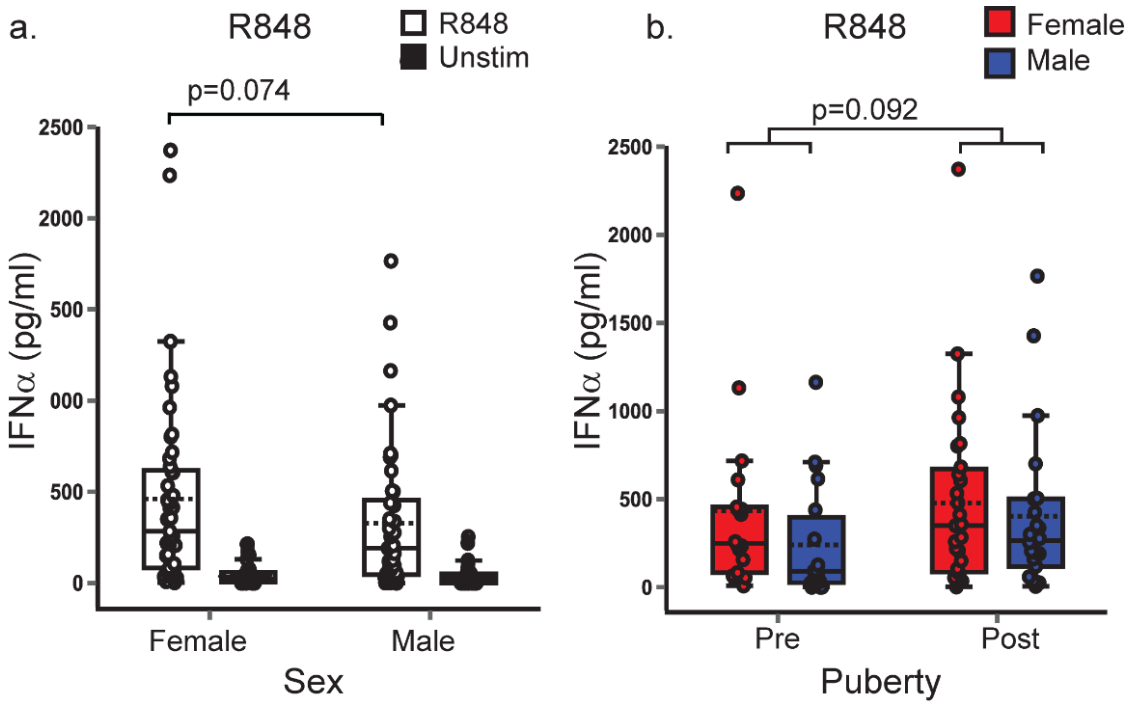


Figure 5-5. There was a non-significant trend towards a higher production of IFNα by PBMC in females and post pubertal volunteers after R848 stimulation. After stimulation with R848, the amount of IFNα produced by PBMC was measured and analysed by linear regression in healthy volunteers (n=86, Pre F=18, Pre M= 19, Post F=27, Post M=22). There was a non-significant trend towards higher production in a. female (p=0.074) and b. post pubertal volunteers (p=0.081). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

5.1.6. Sex or pubertal phase did not associate with the production of IFNα after TLR9 stimulation in healthy young volunteers.

There were no sex (B=-1.601; p=0.535; CI=-6.711-3.509) or pubertal (B=3.681; p=0.162; CI=-1.510-8.872) differences in the percentage of pDC producing IFNα in healthy volunteers after cells were stimulated with CpG (Fig. 5-6 a,b). Similarly, there were no associations between sex and puberty and the production of IFNα (Fig. 5-6c), IFNβ or TNFα by PBMC after CpG stimulation.

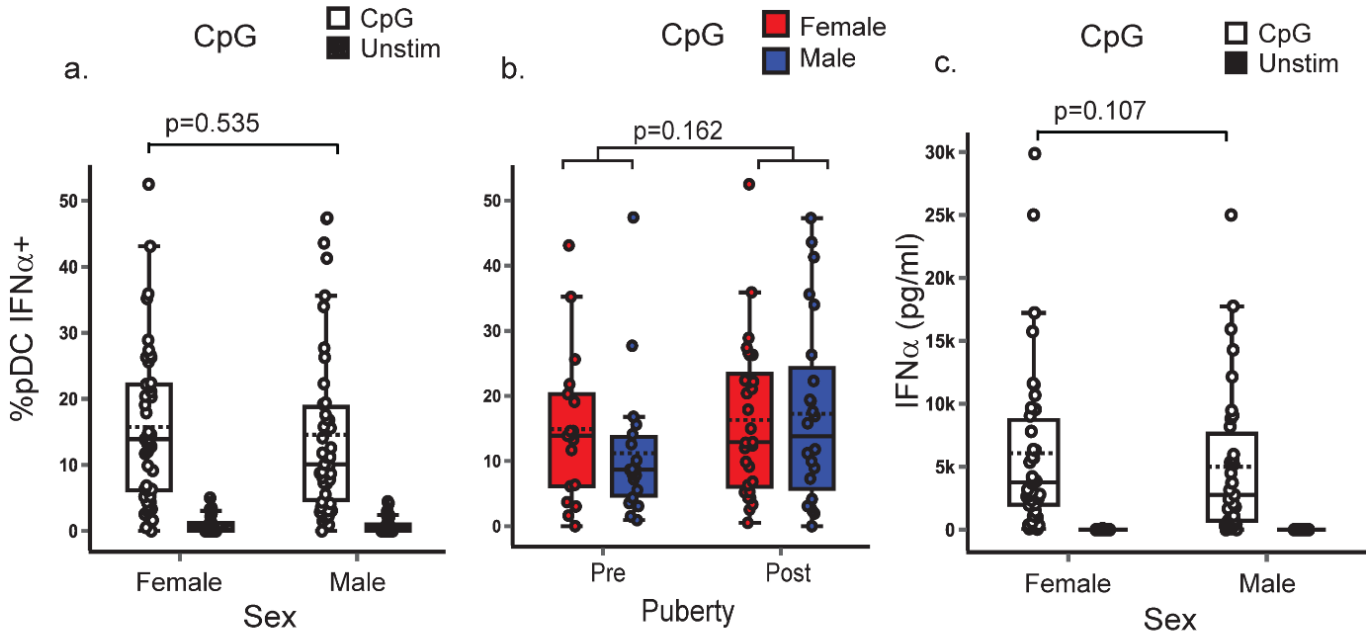


Figure 5-6. Sex or pubertal phase did not associate with the production of IFNα after TLR9 stimulation in healthy young volunteers. After stimulation with CpG, the percentage of pDC producing IFNα was measured and analysed by linear regression (Pre F=18, Pre M=19, Post F=28, Post M =25). There were no significant differences between a. sex (p=0.535) or b. pubertal phase (p=0.162). c. There was no sex difference in the amount of IFNα produced by PBMC after stimulation with CpG(Pre F=15, Pre M=17, Post F=24, Post M =18). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

5.2. X chromosome and sex hormone associations with TLR mediated production of IFNα and other cytokines.

Aim: To investigate whether the percentage of pDC producing IFNα after R848 or CpG stimulation associates with X chromosome number, serum oestradiol and testosterone concentration in healthy, transgender and TUS volunteers.

Sub Aim: To investigate whether the PBMC production of IFNα, IFNβ and TNFα, after R848 or CpG stimulation associates with X chromosome number, serum oestradiol and testosterone concentration in healthy, transgender and TUS volunteers.

5.2.1. TLR7 induced pDC IFNα production associated significantly with X chromosome number and serum testosterone depending on X chromosome number.

Transgender volunteers, volunteers with TUS and healthy volunteers were included to investigate for associations between the percentage of pDC producing IFNα after R848 stimulation, X chromosome number, serum oestradiol and testosterone concentrations.

A linear regression model was fitted. The most parsimonious model, which accounted for the most variation in the percentage of pDC producing IFNα, is represented below. It included serum testosterone concentration, X chromosome number, an interaction term between the two, and serum oestradiol concentration.

*%pDC IFNα+= Intercept + B_1*X chromosome + B_2*Testosterone + B_3*X chromosome*Testosterone + B_4*Oestradiol*

The coefficients, significance values and confidence intervals for the model are represented below in Table 5-2. Overall, this model accounted for 10 % of the variation in the percentage of pDC producing IFNα and was statistically significant (p=0.012). If two X chromosomes were present, after controlling for sex hormone concentrations, there were on average 12.41% more pDC producing IFNα after R848 stimulation (B=12.407; p=0.003; CI=4.434, 20.380) compared to if one X was present (Fig. 5-7a). The model included a significant association between the percentage of pDC producing IFNα after R848 stimulation and serum testosterone (B=0.740; p=0.008; CI=0.197, 1.284) and a significant interaction term between X chromosome number and serum testosterone (B=-1.315;p=0.002; CI=-2.144, -0.486), which improved the predictive value of the model (an illustration of this association, when oestradiol is held constant at 5pmol/L, is given in Fig. 5-7b). This implies that there is a positive association between the percentage of pDC producing IFNα after R848 stimulation with testosterone in the presence of one X, and a negative association in the presence of two X chromosomes. This implies that females with high testosterone and males with low testosterone should both have a decreased TLR7 induced pDC IFNα production when compared to their chromosomal counterparts. To demonstrate this, an ANOVA with a Bonferroni adjusted post-hoc test confirmed that there was a lower percentage of IFNα-producing pDC in trans males (birth female-two X chromosomes; high testosterone) compared to healthy females (mean difference=15.23; p=0.018; 95% CI=1.75, 28.72) and trans females (birth males-one X chromosome, low testosterone) when compared to healthy males (mean difference=17.41; p=0.047; 95% CI=0.16, 34.66) (Fig. 5-7c). There was no association between serum oestradiol concentration and the percentage of pDC producing IFNα after R848

stimulation (B=0.006; p=0.567; CI=-0.014, 0.025), but the inclusion of oestradiol in the model improved the predictive value of the model (adjusted r^2).

Dependant variable: % pDC IFNα+ after R848 stimulation Adjusted r^2=0.101; Model significance p=0.012				
Variables	B coefficient	p	95.0% Confidence Interval for B	
(Constant)	14.753	0.000	8.568	20.938
X chromosome number (1=0; 2=1)	12.407	0.003**	4.434	20.380
Testosterone nmol/L	0.740	0.008*	0.197	1.284
Test/X interaction	-1.315	0.002**	-2.144	-0.486
Oestradiol pmol/L	0.006	0.567	-0.014	0.025

Table 5-2. Full linear regression model assessing for associations between X chromosome number; sex hormone and the percentage of pDC producing IFNα after R848 stimulation in healthy (pre and post puberty), Turner's and transgender volunteers.* p value <0.05; **p value <0.005

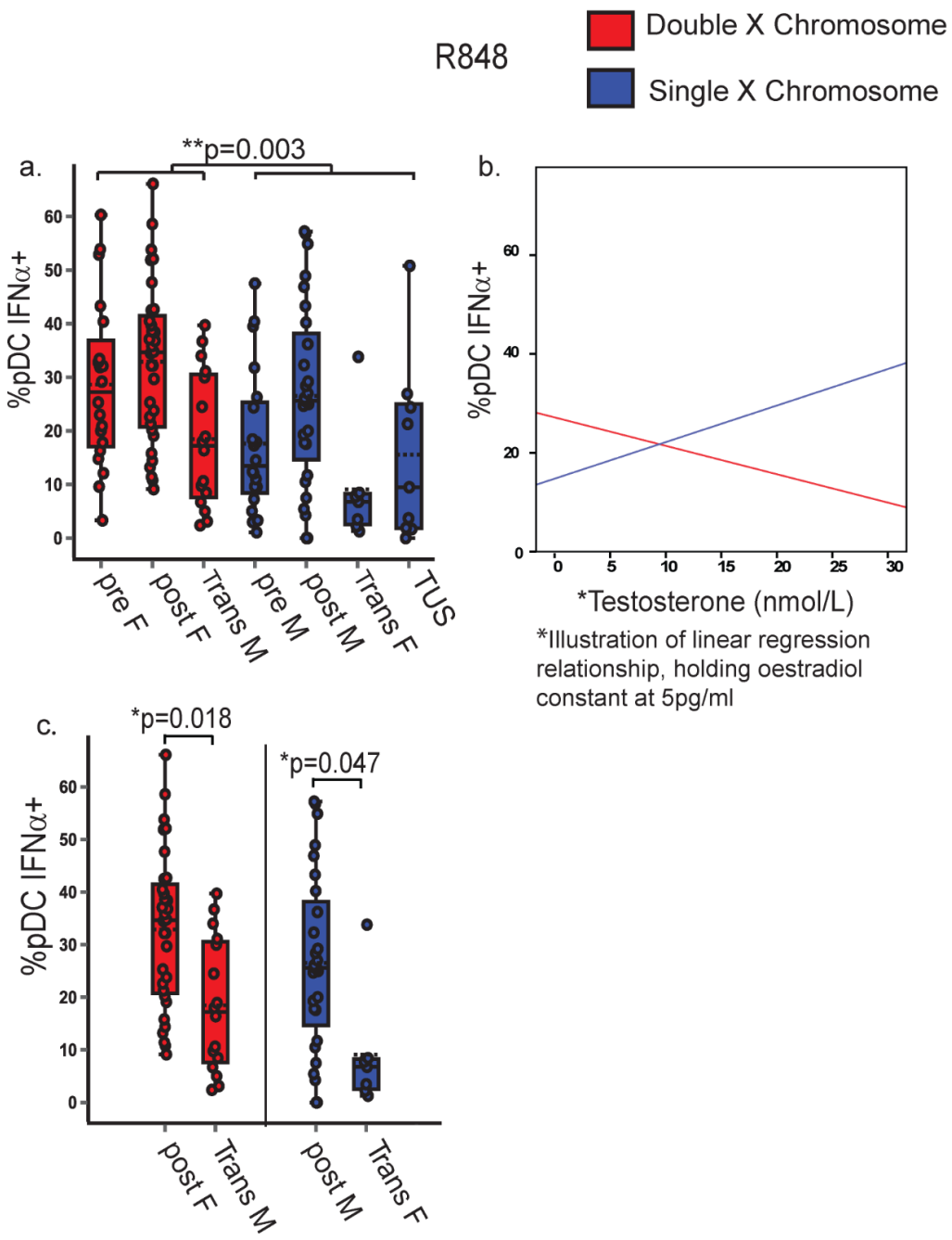


Fig. 5-7. The percentage of pDC producing IFNα after R848 stimulation associated with X chromosome number and serum testosterone depending on X chromosome number. Pre- and post-pubertal healthy, transgender and TUS volunteers were analysed (n=128 Pre F=20, Post F=31, Trans M=13, Pre M=20, Post M=28, trans F=7, Tus=9), p values in a. represent the significance of the coefficient the variable shown as estimated by linear regression after correcting for the other variables in the model (X chromosome number, serum testosterone and oestradiol). a. If two X chromosomes were present, a higher % pDC produced IFNα+ after R848 stimulation (p=0.003). b. % pDC IFNα+ after R848 stimulation associated with serum testosterone (p=0.008) with a significant interaction term between X chromosome number and serum testosterone

(p=0.002) as illustrated, *holding oestradiol constant at 5pmol/L. c. Trans males (p=0.018) and females (p=0.047) both had a lower %pDC IFNα+ after R848 stimulation than their chromosomal counterparts when analysed by ANOVA with post hoc analysis.

5.2.2. PBMC IFNα and IFNβ production associated with X chromosome number, and serum testosterone depending on X chromosome number.

To test the pDC-specific findings above, the production of cytokine in supernatant by PBMC was measured in healthy, transgender and TUS volunteers. The same regression model as in 5.2.1. was fitted using the natural log of the total PBMC production of IFNα or IFNβ after R848 stimulation as the outcome variable (n=111). Please see Tables 5-3 and 5-4 for coefficients, significance values and confidence intervals.

After R848 stimulation, if two X chromosomes were present, after controlling for sex hormones, there was on average 2.8 times more IFNα (ExpB=2.84; p=0.017; CI=1.21-6.67(Fig. 5-8a) and 2.9 times more IFNβ (ExpB=2.94;p=0.030; CI=1.115-7.791)(Fig. 5-8c) produced by PBMC than if one X chromosome was present. In addition, after R848 stimulation, serum testosterone concentration associated with PBMC IFNα (ExpB=1.08; p=0.032; CI=1.01-1.14) and IFNβ production (ExpB=1.08; p=0.030; CI=1.00-1.16). There were significant interaction terms between serum testosterone and X chromosome number with IFNα (ExpB=1.12; p=0.027; CI=0.80-0.98) (Fig. 5-8b) and IFNβ (B=0.875; p=0.019; CI=0.086-1.023) (Fig. 5-8d) concentrations.

Serum sex hormone or X chromosome number did not associate with the production of TNFα in supernatant by PBMC after R848 stimulation.

Dependant variable: PBMC IFNα (pg./ml) production after R848 stimulation Adjusted r^2=0.101; Model significance p=0.024				
Variables	ExpB coefficient	p	95.0% Confidence Interval for ExpB	
(Constant)	84.77	0.000	43.68	164.35
X chromosome number (1=0; 2=1)	2.84	*0.017	1.21	6.66
Testosterone nmol/L	1.07	*0.024	1.01	1.14
Oestradiol pmol/L	0.99	*0.047	0.99	1
Test/X interaction	0.89	*0.028	0.81	0.99

Table 5-3. Full linear regression model assessing for associations between X chromosome number; sex hormone and the PBMC production of IFNα after R848 stimulation in healthy (pre and post puberty), TUS and transgender volunteers (n=111). * p value <0.05; **p value <0.005

Dependant variable: PBMC IFNβ (IU/ml) production after R848 stimulation Adjusted r^2=0.049; Model significance p=0.115				
Variables	ExpB coefficient	p	95.0% Confidence Interval for ExpB	
(Constant)	5.51	0.000	2.59	11.75
X chromosome number (1=0; 2=1)	2.94	0.030	1.11	7.79
Testosterone nmol/L	1.08	0.030	1.00	1.16
Oestradiol pmol/L	0.99	0.634	0.99	1.00
Test/X interaction	0.87	0.019	0.78	0.97

Table 5-4. Full linear regression model assessing for associations between X chromosome number; sex hormone and the PBMC production of IFNβ after R848 stimulation in healthy (pre and post puberty), Turner's and transgender volunteers (n=111)

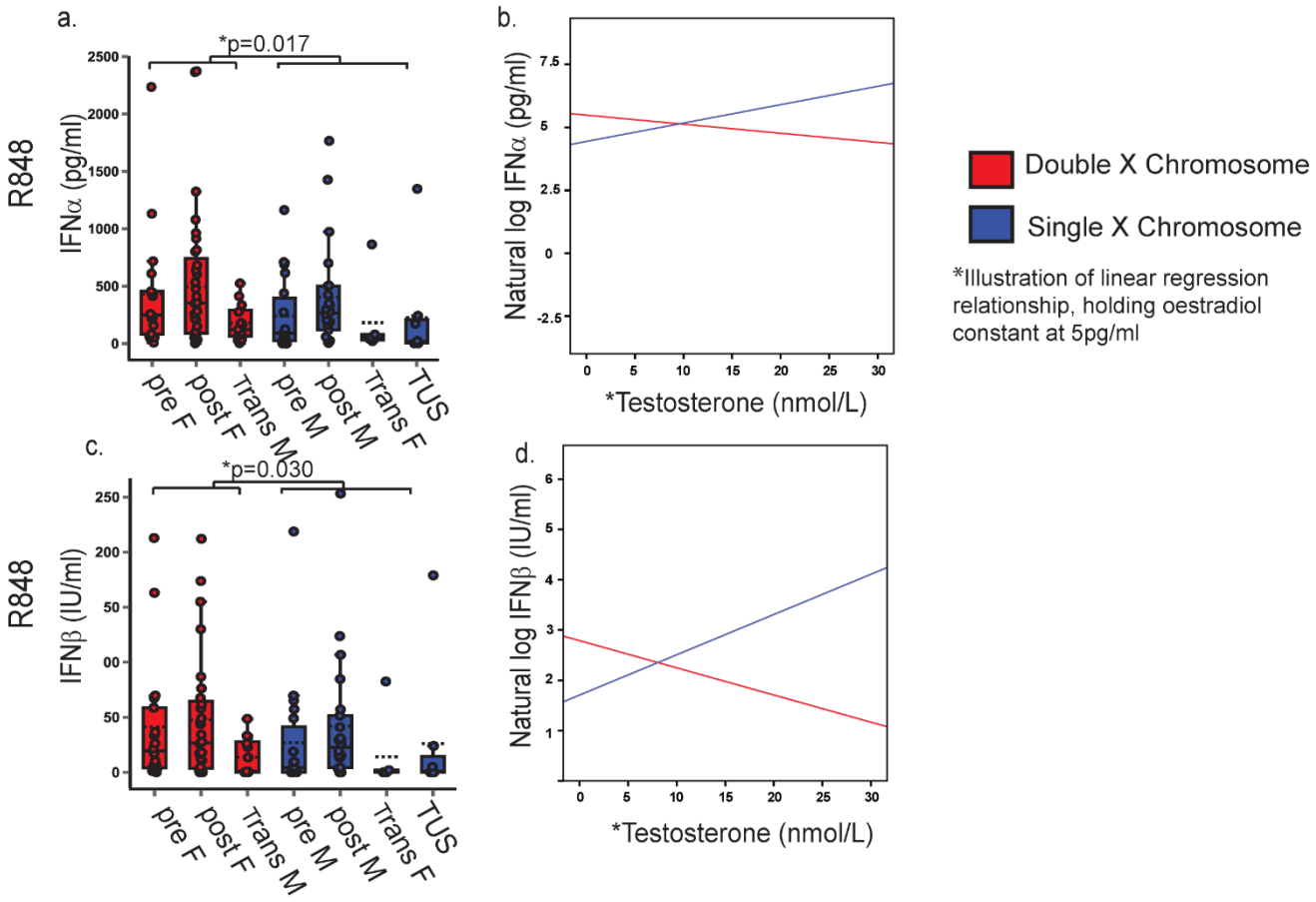


Figure 5-8. X chromosome number and serum testosterone concentration have a significant and interactive association with TLR7 induced PBMC IFNα and IFNβ production. PBMC from healthy, transgender and TUS volunteers were stimulated with R848 (TLR7/8) and concentrations of IFNα, IFNβ and TNFα were measured in supernatant by luminex, p values represent significance of the coefficient for the independent variable shown (n=111 Pre F-18, Post F=27, Trans M=11, Pre M=19, Post M=22, Trans F=6, TUS=8). a.-d. After R848 stimulation, if two X chromosomes were present, PBMC produced more IFNα (p=0.017) and IFNβ (p=0.03) with a significant interaction term between testosterone and X chromosome number (p=0.028; p=0.019 respectively).

5.2.3. TLR9 induced pDC IFNα production associated with serum testosterone concentration, but not X chromosome number.

After CpG stimulation, there was no significant association between the percentage of pDC producing IFNα and X chromosome number (p=0.243) (Fig. 5-9a). There were no associations between the percentage of pDC producing IFNα and X chromosome number or serum oestradiol, but an association existed with serum testosterone concentration (B=0.491; p=0.007; CI=0.138, 0.845) with no significant interaction term (Fig. 5-9b). After CpG stimulation, there were no significant associations with the total PBMC supernatant

production of IFNα, IFNβ or TNFα and X chromosome number or serum sex hormone concentration.

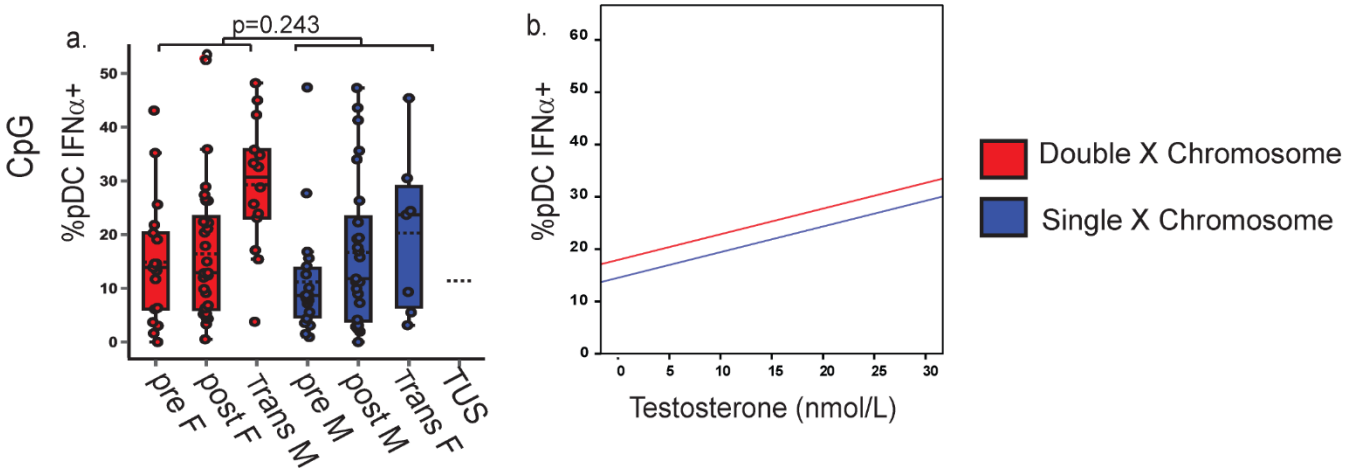


Fig. 5-9. The percentage of pDC producing IFNα after CpG stimulation associates with serum testosterone concentration. PBMC from healthy, transgender volunteers and TUS volunteers were stimulated with CpG (TLR9 agonist) and pDC IFNα production was measured by flow cytometry (n=90 Pre F=18, Post F=28, Trans M=9, Pre M=19, Post M=25, Trans F=11, TUS=9). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression. a. After CpG stimulation, there were no associations between the percentage of pDC producing IFNα and X chromosome number (p=0.243). b. A significant association existed with serum testosterone concentration (p=0.007) with no significant interaction terms.

5.3. Differences in TLR mediated production of IFNα and other cytokines between healthy young people and those with jSLE.

Aim: To investigate whether differences exist in the TLR7 or TLR9 mediated production of IFNα by pDC between healthy young people and those with jSLE.

Sub Aim: To investigate whether differences exist in the TLR7 or TLR9 mediated PBMC production of IFNα, IFNβ and TNFα between healthy young people and those with jSLE.

5.3.1. TLR7 mediated production of IFNα by pDC was not different in jSLE and maintained a sex bias.

The percentage of pDC producing IFNα after R848 stimulation was compared between young people with jSLE and healthy post pubertal volunteers by t-test and no difference was seen(p=0.632) (Fig. 5-10a).

In 5.1.4. it was shown that female pDC in healthy young people had a higher percentage of pDC producing IFNα after TLR7 stimulation than males. It was found that in jSLE, there was still a non-significant trend towards females having a higher percentage of pDC producing IFNα after TLR7 stimulation (t-test p=0.057) (Fig. 5-10a).

5.3.2. TLR7 mediated PBMC production of TNFα but not IFNα or IFNβ was higher in jSLE.

As mentioned above, the distribution of the supernatant cytokine levels was not normal, so non-parametric measures (Mann Whitney U test) to compare between 2 groups were used.

There was no difference in R848 induced PBMC production of IFNα in supernatant between healthy, post pubertal volunteers and those with jSLE (p=0.784) (Fig. 5-10b). In addition, there was no difference in the R848 induced PBMC production of IFNβ (p=0.699) (Fig. 5-10c). Volunteers with jSLE had a higher production of TNFα after stimulation with R848 compared to healthy post pubertal volunteers (p=0.001) (Fig. 5-10d).

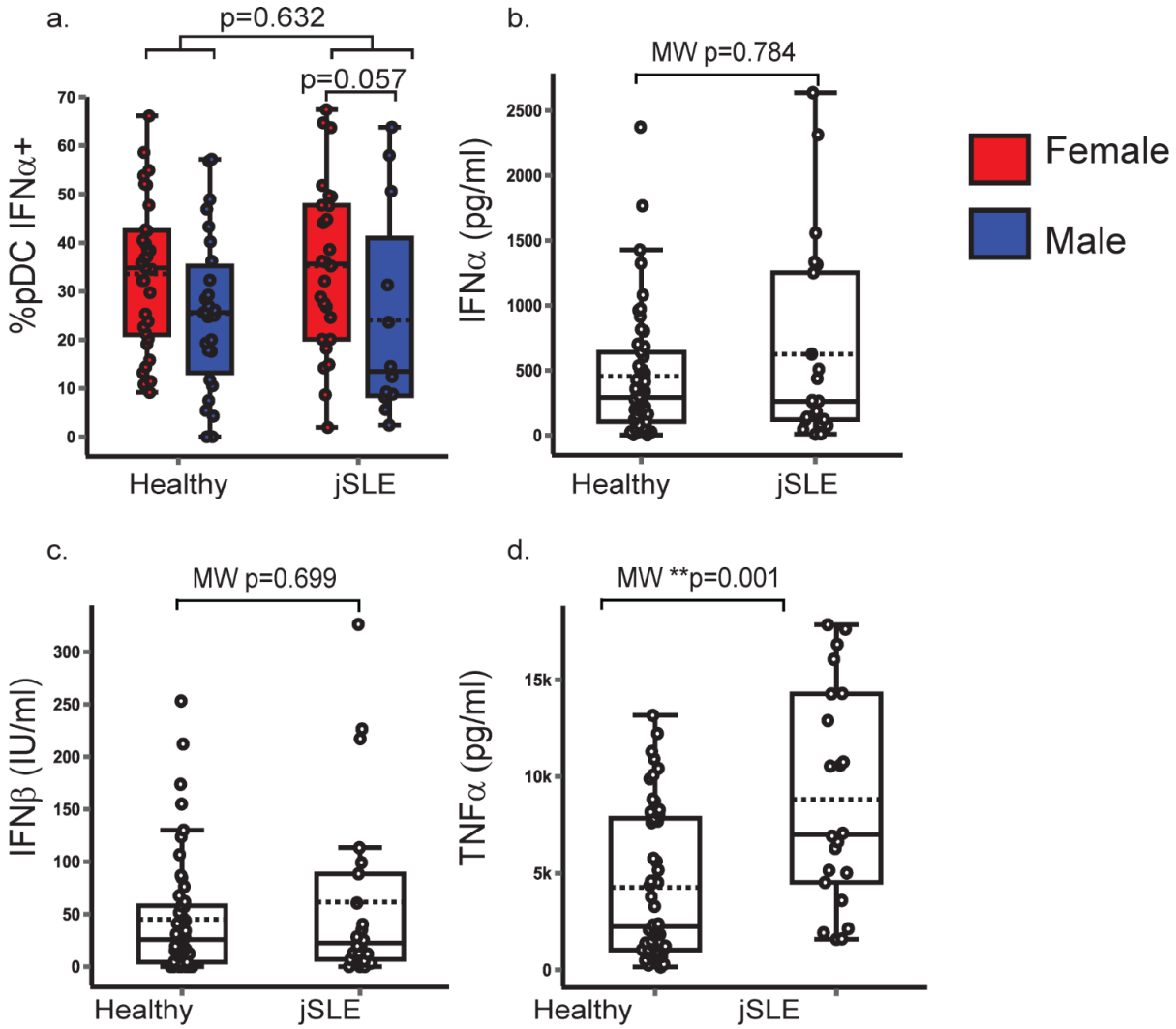


Figure 5-10. TLR7 Induced cytokine production in jSLE a. After R848 stimulation, there was no significant difference in the %pDC producing IFNα in jSLE (t-test: p=0.632, n=98-healthy-F=31, M=28; jSLE F=26, M=13). Females with jSLE had a trend towards a higher % pDC producing IFNα than males with jSLE (p=0.057). b.-c. There were no differences in jSLE in the amount of IFNα (p=0.784) or IFNβ (p=0.699) produced by PBMC in supernatant after R848 stimulation d. After R848 stimulation here was more TNFα produced in jSLE (p=0.001, n=108). MW=Mann Whitney U test

5.3.3. TLR9 induced cytokine production was decreased in jSLE.

In volunteers with jSLE, there was a lower percentage of pDC producing IFNα after TLR9 stimulation when compared to healthy volunteers (p=0.001) (Fig. 5-11a). In addition, PBMC from volunteers with jSLE produced less IFNα (p=0.002), IFNβ (p=0.003) and TNFα (p=0.001) than healthy controls after CpG stimulation (Fig. 5-11b-d).

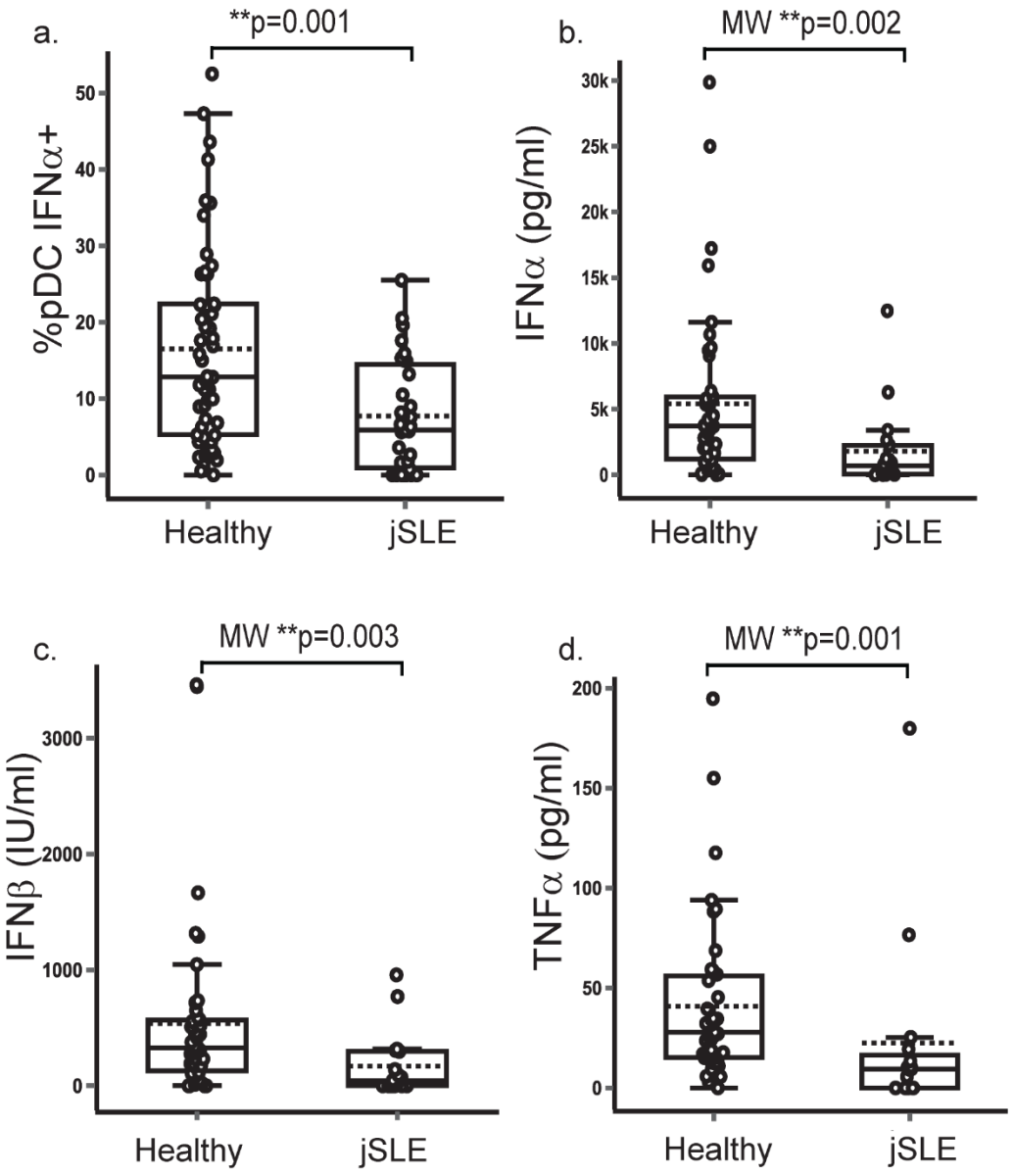


Figure 5-11. TLR9 induced cytokine production is decreased in jSLE. Cells from healthy post pubertal volunteers and volunteers with jSLE were stimulated with CpG (TLR9 agonist). a. The percentage of pDC producing IFNα was lower in jSLE (t test p=0.001, n=90). b.-d. The PBMC production of IFNα (p=0.002); IFNβ (p=0.003) and TNFα (p<0.001) was lower in volunteers with jSLE(n=74). MW=Mann Whitney U test

5.4. Discussion

These are the first data investigating the TLR induced pDC production of IFNα in childhood and across puberty. It has been observed that, upon TLR7 stimulation, a higher percentage of pDC from females produce IFNα than males, regardless of pubertal development. Puberty itself, regardless of sex, associated with a higher percentage of pDC producing IFNα after TLR7 stimulation. TLR7 induced IFNα production associated with serum testosterone concentration, but the direction of this association differed, depending on the number of X chromosomes present. These sex and pubertal differences were unique to TLR7 and not TLR9 induced IFNα production. Importantly, the differences in sex and pubertal phase were unique to the TLR7 induced production of IFNα and IFNβ, and not TNFα, suggesting that this was not a ubiquitous effect on all TLR7 induced cytokines, but that it was specific to type 1 IFNs.

Multiple previous studies have shown that a higher percentage of pDC from adult females produce IFNα after TLR7 stimulation (16, 120, 123, 259). The only non-adult study ,in 2 month old infants, showed that female infants had a higher percentage of pDC producing IFNα after TLR7 stimulation compared to male infants(123). They postulated that this sex difference may have been due to the surge of testosterone, or 'mini-puberty' in male infants in the neonatal period.

To the best of our knowledge this, is the first time that this has been studied in children and adolescents. The current data show that females have a higher percentage of pDC producing IFNα after TLR7 stimulation, regardless of sexual maturity. Sexual maturity however associated with a higher percentage of pDC producing IFNα after TLR7 stimulation, regardless of sex. It is remarkable that in the linear regression model, two essentially demographic variables (sex and pubertal phase) accounted for 10% of the variability in the production of IFNα, a potent cytokine, in response to TLR7 stimulation.

Previous human studies have not been able to separate out the individual effects of sex hormones and X chromosome numbers *in vivo*. When the model in the current study was extended to include volunteers with a variation of serum sex hormone on the background of one or two X chromosomes, it was seen that the TLR7 induced production of IFNα by pDC was substantially and significantly higher if two X chromosomes were present, regardless of serum concentration of testosterone or oestradiol. These findings agree with a humanised mouse model where male or female human progenitor cells were transplanted into male or female NOD-SCID-β2m−/− mice (68). It was seen that regardless of the sex of the recipient mouse, if the transplanted human pDC contained two X chromosomes, a higher percentage of them produced IFNα after TLR7 stimulation.

In our model, it was also noted that the TLR7 mediated pDC production of IFNα associated with serum testosterone concentration, and that the direction of this association was different depending on the number of X chromosomes present. This finding is novel, and it would not have been possible to observe these associations without these unique young volunteers. There have been conflicting reports about the associations between TLR7 mediated pDC function and sex hormones *in vitro* and *in vivo*. *In vitro*, testosterone has been shown to associate with a decrease in the TLR7 induced IFNα production in pDC from adult females, whereas oestradiol, or oestradiol antagonism has had no effect on the *in vitro* assay (16, 124). It has previously been shown that exogenous oestradiol administration in post-menopausal women associated with an increase in TLR7 induced pDC production of IFNα and TNFα, but these data did not take serum testosterone concentration into account as a confounding variable(68). The current data suggest that testosterone has an important association with TLR7 associated pDC IFNα production, but that the direction of this association changes with sex. Testosterone therefore, may be a candidate to investigate, in females specifically, to potentially modulate the TLR7 mediated pDC IFN response.

In these data, pDC only represent 0.1-0.9% of total PBMC. Due to small blood volume and cell numbers obtainable in children and adolescents, it was not possible to confirm the flow cytometry findings by separating sufficient pDC and assessing for the pDC-specific amount of IFNα produced in supernatant. Therefore, although less precise, the amount of IFNα produced in supernatant by total PBMC after stimulation with R848 was measured as confirmation of the pDC specific findings. Despite the main IFNα producing cell type (pDC) representing such a low percentage of cells, and there being fewer numbers with the measure available, there were still non-significant trends towards increased production in females and post pubertal volunteers as had been seen in the pDC specific experiment. When the model was extended and the associations with X chromosome number and sex hormone were analysed, the production of IFNα and IFNβ but not TNFα associated significantly with X chromosome number and serum testosterone as they had done in the pDC specific experiment. In addition, these data confirmed a significant interaction term between X chromosome number and serum testosterone for both IFNα and IFNβ.

It is known that R848 is an agonist of both TLR7 and TLR8. Although the effects of TLR8 stimulation cannot be definitively evaluated from this data, pDC are known to constitutively express TLR7 and not TLR8 and therefore the results of R848 stimulation on pDC are most likely to represent TLR7 stimulation (260, 261). The indirect effect of TLR8 on other cell types however, cannot be excluded.

As there were no sex or pubertal associations with the production of any cytokines after CpG or TLR9 stimulation, we can conclude that this effect was specific to TLR7, and not ubiquitous for nucleic acid sensing by TLRs. As expected after no sex difference was shown, TLR9 induced pDC IFNα production did not associate with X chromosome number but did show an association with serum testosterone concentration. Therefore, put together, in these data, a lower serum testosterone concentration associated with a lower TLR9 mediated pDC IFNα production, regardless of sex, and a higher TLR7 mediated pDC IFNα response in females only.

As has been stated before, jSLE is a disease with a characteristic type 1 IFN signature. It is not clear where the source of this extra IFNα lies. In the current data, there were no difference in the production of IFNα after TLR7 stimulation between healthy volunteers and those with jSLE. In jSLE, as in healthy volunteers, females still had a higher percentage of pDC producing IFNα after TLR7 stimulation. There have been varying reports about whether TLR7 induced IFNα production is different in SLE (35, 244, 245). A recent study involving 71 adult SLE patients and 45 healthy adults showed an increase in the TLR7 mediated pDC production of IFNα and TNFα in SLE(245). This study included patients with active disease in addition to those with low disease activity and reported that the TLR7 mediated pDC production of IFNα correlated with disease activity. It is possible therefore that the present data do not show a difference, partly because the patients were selected to have a low disease activity. Interestingly, the present data do show an increase in TNFα production after TLR7 stimulation in jSLE when compared to healthy volunteers.

The TLR9 induced production of IFNα was markedly decreased in jSLE patients, along with the PBMC production of IFNα, IFNβ and TNFα. This phenomenon has been widely reported before (244, 262, 263). jSLE therefore associates with a significant decrease in TLR9 mediated cytokine production, whereas TLR7 mediated cytokine production is reported to be unchanged or increased with disease activity. In addition, previous data has shown that pre-stimulation of PBMC with IFNα sharply decreases TLR9 mediated cytokine production, but increases TLR7 mediated cytokine production (245).

It may stand to reason, that the appropriate response to a high background IFN would be a compensatory down-regulation in the IFN producing capacity of TLR to prevent a sustained forward feedback loop, as is seen with TLR9 induced IFNα production. Therefore, the observation that TLR7 induced IFNα production is not changed may be of importance. Further work is needed to assess whether this is appropriate or not in the context of jSLE. From these various observations, it might be postulated that the IFNα that drives SLE may be more related to TLR7 stimulation, than TLR9, or at least, that a balance exists between the two, which is

disrupted in jSLE. This makes the above observation of the effects of serum testosterone intriguing. In females, testosterone associates with an increase in TLR9 induced IFNα production, and a decrease in TLR7 induced IFNα production, which would make it a logical target to reverse the imbalance of these responses that is seen in jSLE.

These functional results should be viewed with caution, as all of the patients with jSLE were prescribed HCQ, which has the potential to interfere with TLR9 and TLR7 induced cytokine production in a manner that is not fully understood *in vivo* (158, 264). HCQ is almost universally used in jSLE and often started at diagnosis. Patients naïve to all treatment are difficult to access, therefore it is extremely challenging to obtain such samples and perform these experiments in the absence of HCQ.

5.5. Conclusion

Females had a higher TLR7 induced pDC IFNα response regardless of puberty. Post pubertal volunteers had a higher TLR7 induced pDC IFNα response regardless of sex. If two X chromosomes were present, there was a higher TLR7 induced pDC IFNα response, regardless of hormonal environment. Testosterone associated with TLR7 induced pDC IFNα response differently, depending on the number of X chromosomes present. These findings were specific to TLR7 and type 1 IFN production.

Patients with jSLE showed a universal decrease in cytokine production after TLR9 stimulation, but no difference with TLR7 stimulation.

It was next investigated whether the expression of key genes in the type 1 IFN production pathway associated with sex, puberty, X chromosome number or serum sex hormone.

Chapter 6: IFN pathway gene expression, sex and puberty

After observing that female sex and pubertal phase associated with a higher production of IFNα after TLR7 stimulation, and that this associated with X chromosome number and testosterone, it was next investigated whether the PBMC expression of key genes in the IFN sensing and production pathway differed with sex, puberty, X chromosome number or serum sex hormone.

6.1. Gene selection

A gene panel was designed to test for differences in type 1 IFN production pathway. In addition, key gene groups that are known to be over-expressed in jSLE were selected in order to investigate whether these were differentially expressed in sex and puberty in a manner that may predispose females to develop jSLE(26). Genes are given in Table 2-4 in 'Methods'.

6.1.1. IFN score

Five IFN inducible genes were selected to calculate an 'IFN score' (265).

IFN score = (MX1+BST2+IFIT1+ISG15+MCP1)/5

As these genes are expressed at varying levels, it was assessed whether normalising them prior to calculating an average influenced the findings. There was no difference in the significance of results, so it was elected to keep the data in its original form and use the average as calculated above and shown in Fig. 6-1. There was one outlier among healthy volunteers with a high IFN score. This data point came from a 16-year-old female with no evidence of inter-current viral infection on review of her clinical data. Sensitivity analysis was performed, and as this sample was not influential, it was elected to include this outlier in the analysis.

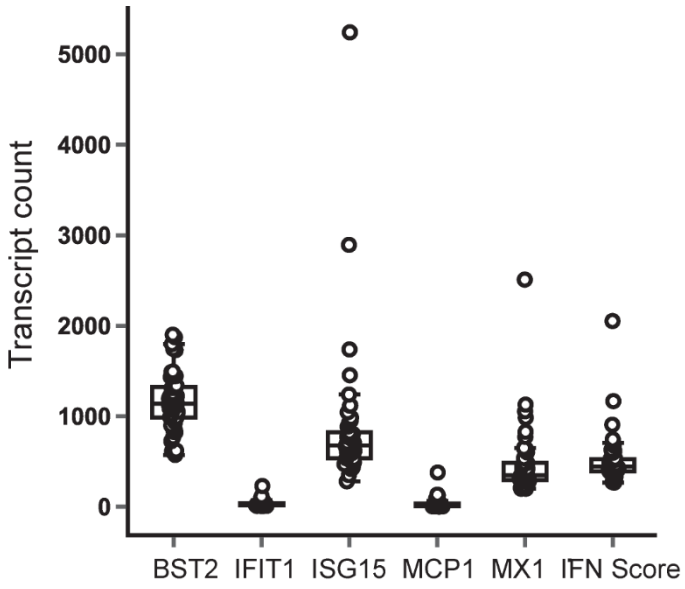


Figure 6-1. IFN Score. Five known IFN inducible genes were measured by Nanostring and an average score or 'IFN score' was calculated. The single outlier was not influential and kept in the analysis.

6.1.2. IFN pathway genes

Genes were selected from the RNA and DNA sensing and signalling pathway (Fig. 6-2). These included endosomal RNA sensor *TLR7*, intracytoplasmic RNA sensors *MDA5* and *RIG-1*, as well as *MAVS*, a signalling molecule downstream of RIG-1 and MDA5. Endosomal DNA sensor, *TLR9*, and intracytoplasmic DNA sensor, *DDX41*, were selected. *IFN regulatory factors (IRF)5* and *7* were selected.

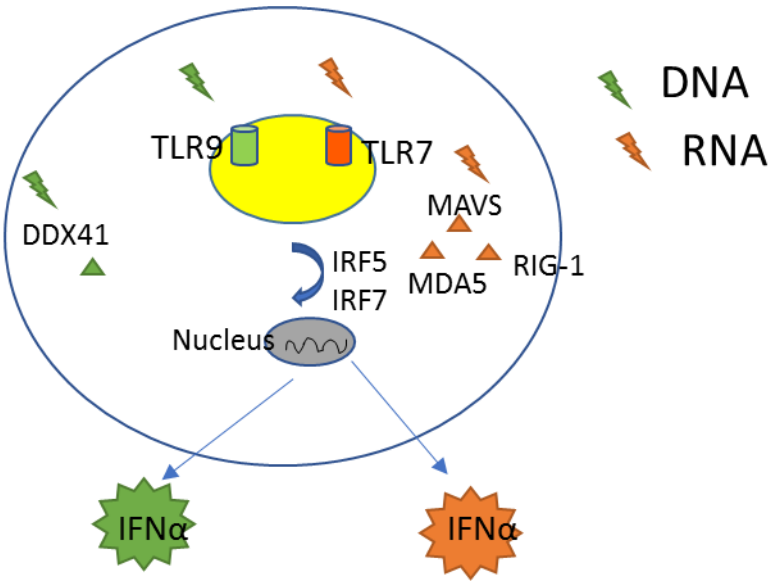


Figure 6-2. Gene selection in the DNA and RNA sensing and signalling pathway.

6.1.3. Other genes

In jSLE, there is increased dead/dying cell debris due to increased apoptosis(266). There is a theory that this provides an increased load of nucleic acid for sensing and IFN production(1). For this reason, *Fas (TNFR6)* and *TNF relate apoptosis inducing ligand* (*TRAIL*) were included in the gene panel.

There has been some investigation into whether ERE nucleic acids may contribute to IFN production(219). *LINE-1* is the most abundant of these elements and is capable of autonomous transposition and has been shown to be differentially expressed in SLE (220). *LINE 1* was found to be the most abundantly expressed gene out of all the genes measured in the selected panel which reflects its ubiquitous nature in cells (Fig. 6-3a). Ro 60 is an RNA binding protein coded by the *TROVE-2* gene that has been shown to bind endogenous retroelement RNA specifically in SLE(267) and was therefore included.

The gene expression of the following hormone receptors was included: oestrogen receptor alpha (*ESR1*); oestrogen receptor beta (*ESR2*); progesterone receptor (*PGR*) and androgen receptor (*AR*). As mentioned in 'Methods', the expression of these genes was not high enough in PBMC to be accurately measured against the background signal (Fig. 6-3b). Therefore, these data were excluded from further investigation.

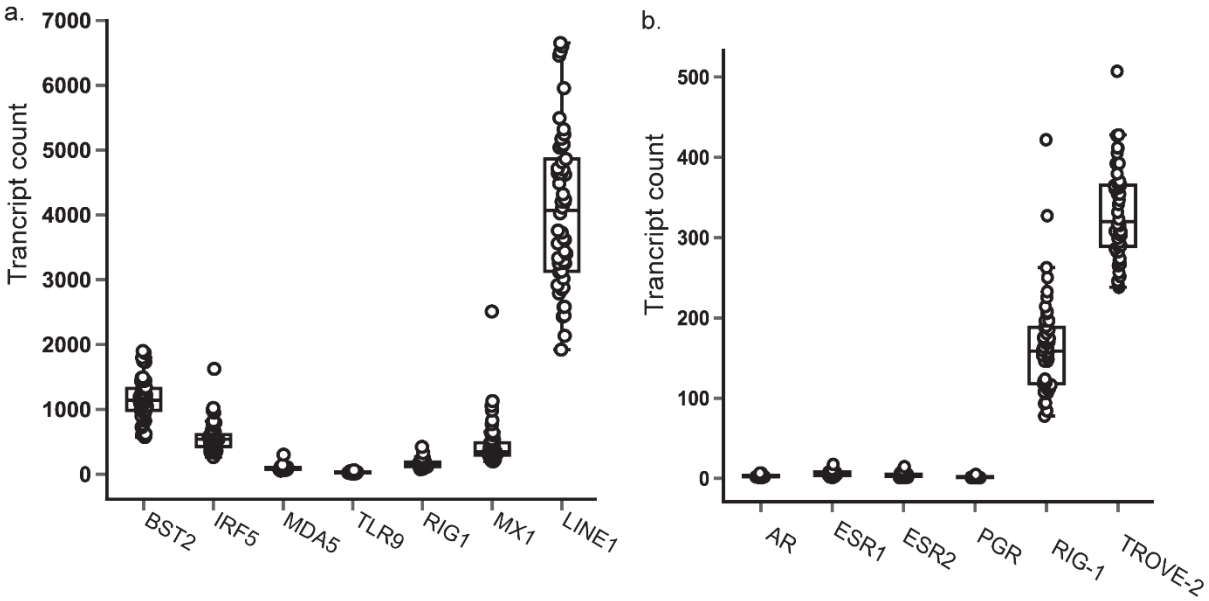


Figure 6-3. Relative gene expression in healthy volunteers. a. The corrected and normalised counts of gene expression were measured in healthy volunteers. It was noted that *LINE1* was the most abundantly expressed gene compared to all others. b. Hormone receptor genes for oestrogen (ESR1/2), progesterone (PGR), and androgen (AR) were measured but not expressed highly enough in PBMC to be accurately measured. These genes are shown here, compared arbitrarily to *RIG-1* and *TROVE* which both had a relatively low expression.

6.1.4. Statistical analysis

All the genes measured were analysed by linear regression to assess for associations between PBMC gene expression with sex and pubertal phase. A summary of all the models for sex and pubertal phase is represented in Table 6-1. In addition, the significance values for each coefficient (B) was adjusted for multiple testing using the Benjamini Hochberg method, with a false discovery rate of 0.25, as shown in Table 6-2.

	Model: $y=intercept+B_1*Sex+B_2*Puberty$										
Investigation	Dependant variable	Model predictive value	Model significance	Independent Variables							
				Sex (F=1; M=0)				Puberty (Pre=0; Post=1)			
RNA sensing		Adjusted r^2	p	Coefficient B_1	p	95% CI		Coefficient B_2	p	95% CI	
	TLR7	0.055	0.099	5.749	0.091	-0.952	12.45	5.246	0.122	-1.455	11.947
	RIG-1	0.069	0.07	37.739	**0.001	3.595	71.883	-9.316	0.586	-43.462	24.826
	MDA5	0.07	0.069	22.894	*0.022	3.379	42.41	-0.365	0.97	-19.881	19.151
	MAVS	0.079	0.055	9.243	0.127	-2.741	21.227	-10.354	0.089	-22.338	1.631
DNA sensing	*TLR9*	0.23	0.001	4.566	0.148	-1.676	10.809	-11.156	**0.001	-17.398	-4.913
	DDX41	-0.29	0.733	-6.097	0.462	-22.622	10.427	1.462	0.859	-15.062	17.987
IRF	*IRF5*	0.083	0.05	-34.434	0.219	-90.006	21.139	56.229	*0.047	0.656	111.801
	IRF7	-0.01	0.473	56.23	0.391	-74.445	186.904	63.445	0.334	-67.23	194.12
Cell death	*TNFR6*	-0.023	0.638	15.036	0.352	-17.114	47.185	-0.575	0.971	-32.724	31.575
	TRAIL	-0.02	0.594	25.881	0.672	-96.44	148.203	59.502	0.333	-62.82	181.824
Endogenous retroelement	*LINE1*	0.191	0.003	-342.498	0.267	-973.068	276.072	-1123.129	**0.001	-1747.699	-498.559
	TROVE	0.088	0.043	28.669	0.071	-2.556	59.893	-24.478	0.121	-55.702	6.747
IFN Score	IFN Score	-0.008	0.455	58.776	0.461	-100.47	218.023	87.461	0.274	-71.606	246.888
	Post stim IFN Score	0.316	0.001	-351.992	0.327	-1073.017	369.033	1428.677	**0.001	699.565	2157.789

Table 6-1. Linear regression analysis of all genes for associations with sex and pubertal phase. All genes in the panel were assessed for association with sex and puberty by linear regression and regression models are summarised here.

Coefficient	Coefficient significance	Benjamini-Hochberg significance	Benjamini-Hochberg P-value (<0.25=significant)
RIG-1(Sex)	0.001	**significant**	0.007
TLR9 (pub)	0.001	**significant**	0.007
LINE1 (pub)	0.001	**significant**	0.007
Post stim IFN Score (pub)	0.001	**significant**	0.007
MDA5 (sex)	0.022	**significant**	0.1232
IRF5 (pub)	0.047	**significant**	0.219
TROVE (sex)	0.071	**not significant**	0.283
MAVS(Pub)	0.089	**not significant**	0.283
TLR7 (sex)	0.091	**not significant**	0.283
TROVE (pub)	0.121	**not significant**	0.296
TLR7 (pub)	0.122	**not significant**	0.296
MAVS (pub)	0.127	**not significant**	0.296
TLR9 (sex)	0.148	**not significant**	0.319
IRF5 (sex)	0.219	**not significant**	0.438
LINE1 (sex)	0.267	**not significant**	0.479
IFN Score (pub)	0.274	**not significant**	0.479
Post stim IFN Score (sex)	0.327	**not significant**	0.492
TRAIL (pub)	0.333	**not significant**	0.492
IRF7(pub)	0.334	**not significant**	0.492
TNFR6 (pub)	0.352	**not significant**	0.492
IRF7 (pub)	0.391	**not significant**	0.521
IFN Score (sex)	0.461	**not significant**	0.562
DDX41 (sex)	0.462	**not significant**	0.562
RIG-1 (pub)	0.586	**not significant**	0.684
TRAIL (sex)	0.672	**not significant**	0.752
DDX41 (pub)	0.859	**not significant**	0.925
MDA5 (pub)	0.97	**not significant**	0.971
TNFR6 (pub)	0.971	**not significant**	0.971

Table 6-2. Multiple correction of gene coefficients for sex and puberty. Benjamini Hochberg method of adjusting for multiple testing was used for the significance values of the coefficients for the associations between gene expression, sex and puberty. A false discovery rate of 0.25 was used.

6.2. Sex and pubertal differences in PBMC gene expression

Aim: To investigate whether sex or pubertal phase associate with the expression of selected genes in the IFN pathway in healthy young people.

6.2.1. The expression of genes coding for RNA sensors was higher in females.

In healthy females, PBMC expressed significantly more of the genes for intracytoplasmic RNA receptors *RIG-1* (p=0.001) and *MDA5* (p=0.022) (Fig. 6-4, Table 6-1), after controlling for pubertal phase. There was no significant difference in the expression of *MAVS* with sex or pubertal phase.

When assessed by linear regression, there was a higher expression of endosomal RNA sensor *TLR7*, but this did not reach statistical significance (p=0.091). An ANOVA with post hoc analysis and Bonferroni correction was performed and revealed that post pubertal females had a significantly higher *TLR7* expression than post pubertal males (p=0.024; mean difference=13.922; 95% CI=1.247, 26.596) and pre-pubertal females (p=0.03; mean difference=13.419; 95% CI=0.744, 26.093).

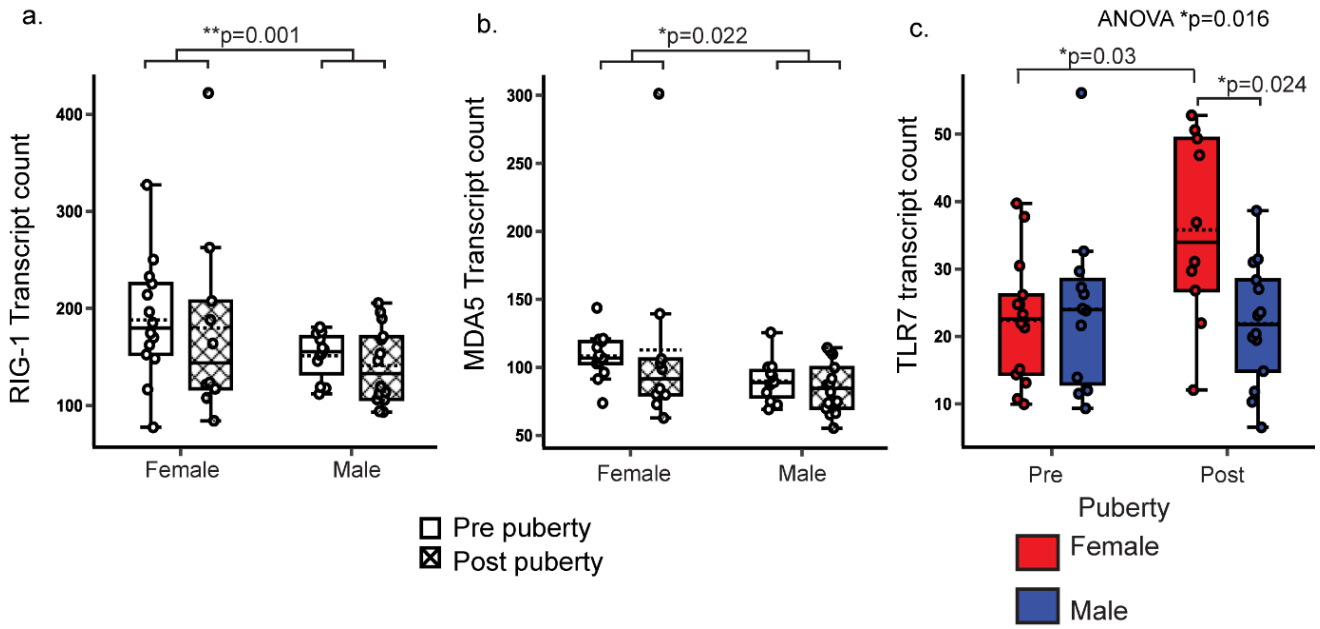


Figure 6-4. RNA sensing gene expression was higher in females. a.-b. The expression of intracytoplasmic RNA sensors *RIG-1* (p=0.001) and *MDA5* (p=0.022) was higher in females than males, after controlling for pubertal phase. c. There was a significantly higher expression of *TLR7* in post pubertal females when compared to post pubertal males (p=0.024) and pre-pubertal females (p=0.03) respectively. (n=50 Pre F=14, Post F=10, Pre M=12, Post M=14). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

6.2.2. *TLR9* gene expression was decreased in post pubertal healthy volunteers.

The PBMC gene expression of endosomal DNA sensor, *TLR9* was decreased in post pubertal volunteers overall (B=-11.156; p=0.001; 95%CI=-17.398, -4.913) with no significant sex difference (Fig. 6-5a, Table 6-2). There were no significant sex or pubertal differences in the expression of intracytoplasmic DNA sensor *DDX41* (Fig. 6-5b, Table 6-1).

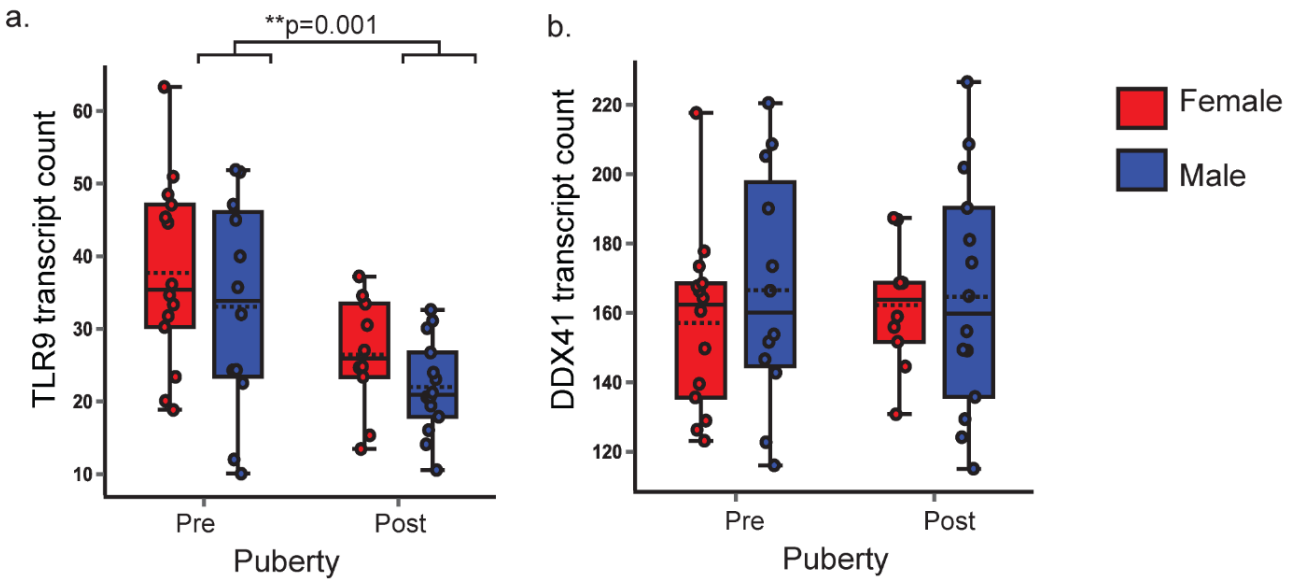


Figure 6-5. PBMC TLR9 gene expression was decreased in post pubertal healthy volunteers. a. *TLR9* gene expression was decreased after puberty when controlling for sex (p=0.001). b. DDX41 gene expression was not significantly different between sexes or pubertal phases. (n=50 Pre F=14, Post F=10, Pre M=12, Post M=14). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression.

6.2.3. *IRF5* gene expression was increased in post pubertal volunteers

In healthy volunteers, there was an increased PBMC gene expression of *IRF5* after puberty (B=56.229; p=0.047; 95%CI=0.656, 111.801) with no significant sex difference (Fig. 6-6a, Table 6-1). There were no significant differences in the PBMC gene expression of *IRF7* with sex or puberty (Fig 6-6b).

6.2.4. *LINE1* gene expression was decreased in post pubertal volunteers

In healthy volunteers, PBMC *LINE1* gene expression was decreased in post pubertal volunteers (B=-1123.129; p=0.001; 95%CI=-1747.699, -498.559) with no significant sex difference (Fig. 6-7, Table 6-1). There were no significant sex or pubertal differences in the PBMC gene expression of *TROVE2* (Figure 6-7b).

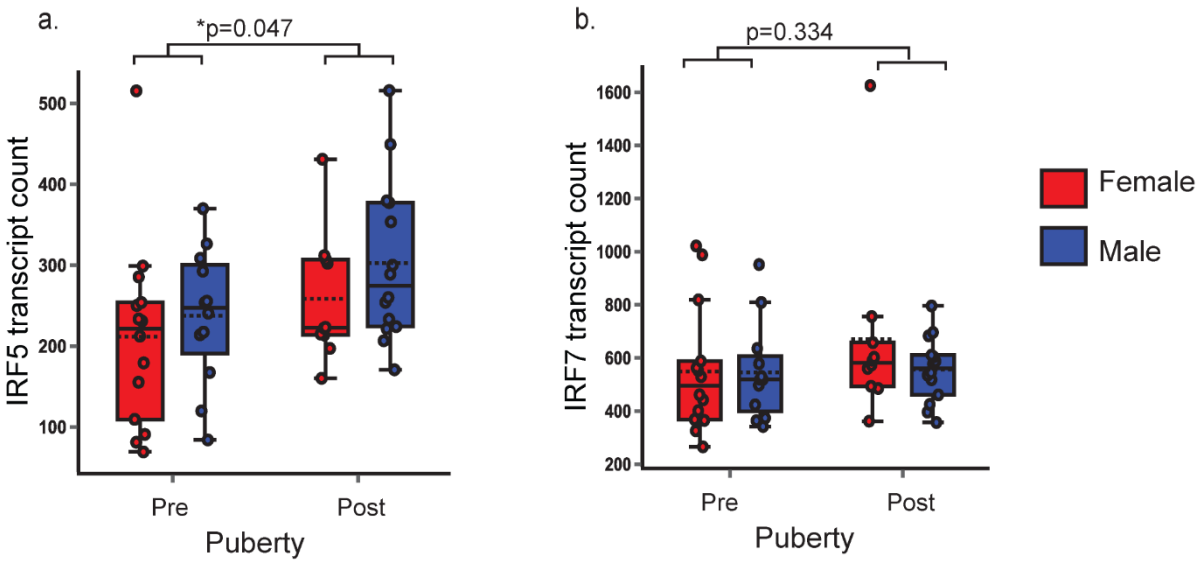


Figure 6-6. IRF5 gene expression was increased in post pubertal volunteers a. In post pubertal volunteers, PBMC *IRF5* gene expression was increased (p=0.047) with no sex difference. b. No significant sex or pubertal differences were seen in PBMC *IRF7* gene expression. (n=50 Pre F=14, Post F=10, Pre M=12, Post M=14)

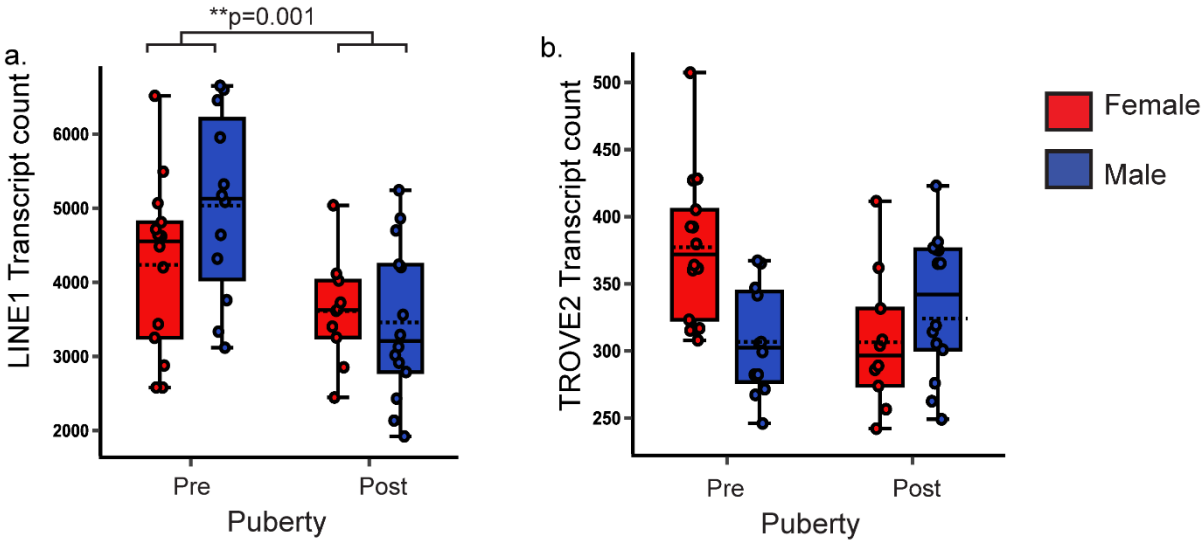


Figure 6-7. LINE1 gene expression was decreased in post pubertal volunteers. a. Gene expression of *LINE1* was decreased in post pubertal healthy volunteers (p=0.001) with no significant sex difference. b. There was no significant sex or pubertal differences in the PBMC expression of *TROVE2*. (n=50 Pre F=14, Post F=10, Pre M=12, Post M=14). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

6.2.5. Cell death associated genes did not associate with sex or pubertal phase.

There were no significant differences in PBMC gene expression of cell death associated genes, *TNFR6* or *TRAIL* between sexes or pubertal groups (Table 6-1).

6.2.6. The upregulated IFN score was higher in post pubertal volunteers.

There was no difference in the *ex-vivo* baseline PBMC IFN score between sexes or pubertal phases (Fig. 6-8a, Table 6-1). PBMC were stimulated with IFNα for 20 hours and IFN score re-measured (upregulated IFN score). There was a significantly higher upregulated IFN score in post pubertal volunteers (B=1428.677; p=0.001; 95% CI=699.565, 2157.789) with no significant sex difference (Fig. 6-8b, Table 6-1). The adjusted r^2 for this model was 0.316, implying that sex and pubertal phase accounted for 32% of the variability seen in the ability of PMBC to upregulate the expression of these ISG upon stimulation with IFNα.

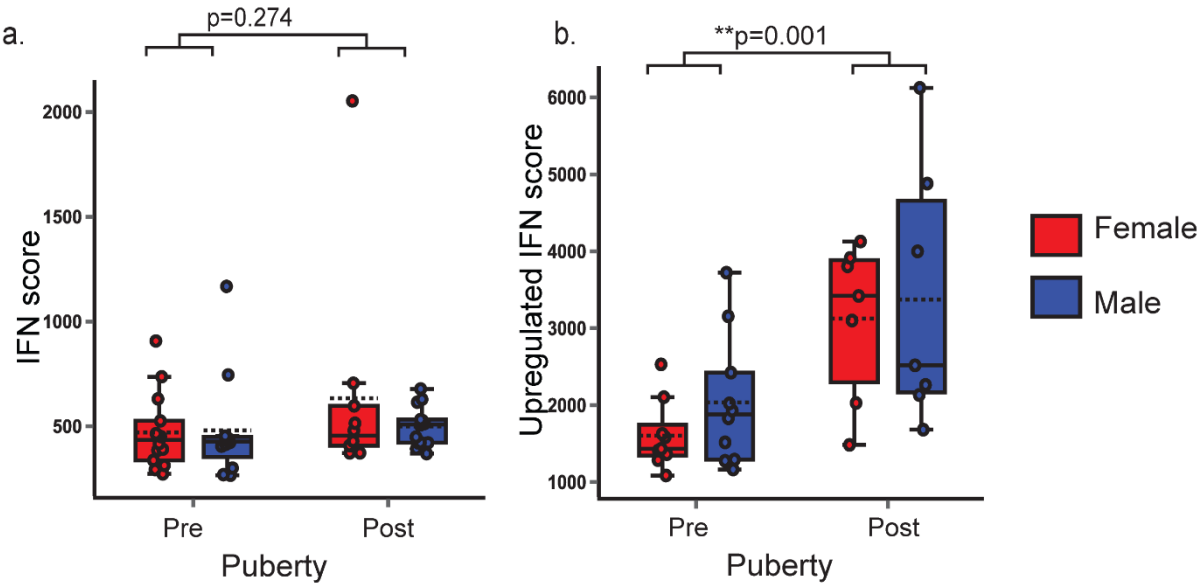


Figure 6-8. After exposure to IFNα, the IFN score is higher in post pubertal volunteers. a. The baseline IFN score was not significantly different between sexes or pubertal phases in healthy volunteers (n=50 Pre F=14, Post F=10, Pre M=12, Post M=14). b. After IFNα stimulation the IFN score (upregulated IFN score) was significantly higher in post pubertal volunteers (p=0.001, n=33 Pre F=9, Post F=7, Pre M=10, Post M=17). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

6.3. X chromosome and sex hormone associations with PBMC gene expression of IFN pathway genes.

After observing that the expression of various genes associated with sex and puberty in healthy volunteers, transgender and TUS volunteers were included to test for associations between selected PBMC gene expression, X chromosome number, and serum sex hormone.

Aim: To test for associations between the PBMC gene expression of *TLR7; RIG-1; MDA5; TLR9; LINE1* and upregulated IFN score with X chromosome number, serum oestradiol and serum testosterone concentration.

6.3.4. The PBMC gene expression of RNA sensor *RIG-1*, but not *MDA5* or *TLR7*, associated with X chromosome number after controlling for sex hormone.

The expression of *RIG-1* was higher if two X chromosomes were present (B=37.342, p = 0.037; 95% CI=2.272, 72.412) with no association with the concentration of either sex hormone (Fig. 6-9a). PBMC gene expression of either *TLR7* or *MDA5* did not associate with X chromosome number, serum testosterone or oestradiol concentration (Fig. 6-9b-c). Regression models summarised in Table 6-3.

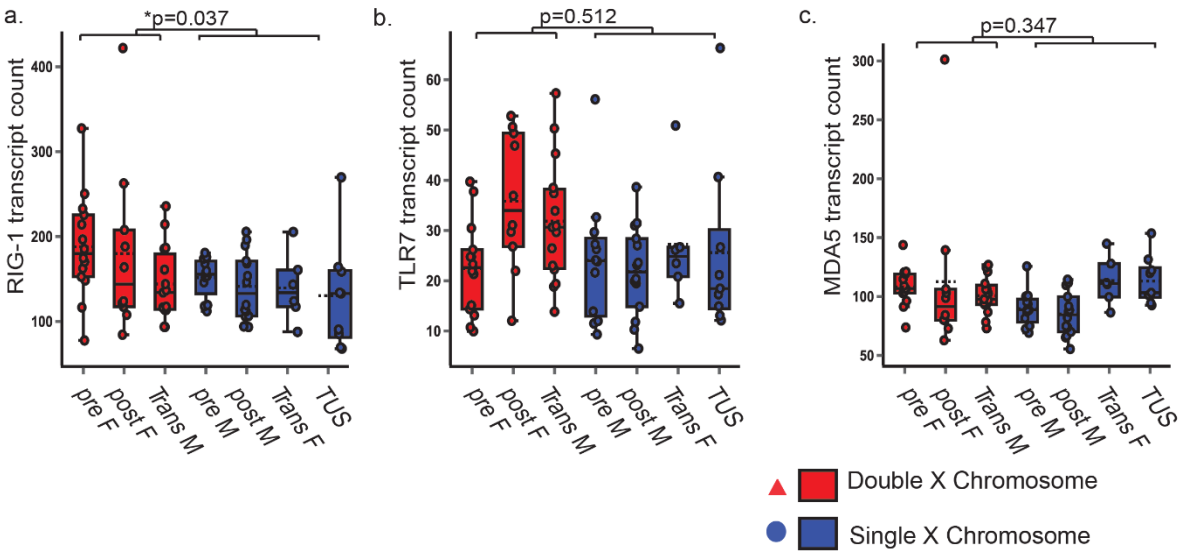


Figure 6-9. PBMC gene expression of *RIG-1* was higher if two X chromosomes were present. a. *RIG-1* gene expression was higher if two X chromosomes were present (p=0.037) regardless of serum sex hormone concentration. b.-c. *TLR7* and *MDA5* gene expression did not associate significantly with X chromosome number or serum sex hormone concentration. (n=77, Pre F=14, Post F=10, Trans M=12, Pre M=12, Post M=14, Trans F=6, TUS=9). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

Model: y=Intercept+B_1*X chromosome number+B_2*Testosterone+B_3*Oestradiol												
Independent Variables	Dependant variables											
	TLR7				*RIG-1*				*MDA5*			
	B	p	95% CI		B	p	95% CI		B	p	95% CI	
X chromosome number (1=0; 2=1)	2.497	0.512	-5.091	10.084	37.342	*0.037	2.272	72.412	8.633	0.347	-9.653	26.919
Testosterone nmol/L	0.021	0.92	-0.398	0.441	-0.782	0.421	-2.722	1.157	-0.777	0.129	-1.788	0.234
Oestradiol (pmol/L)	0.001	0.926	-0.02	0.022	-0.029	0.562	-0.127	0.07	0.028	0.284	-0.024	0.079

Table 6-3. Linear regression models testing for associations between RNA sensors, X chromosome number and serum sex hormone in healthy and transgender volunteers and volunteers with TUS.

6.3.5. The PBMC gene expression of *TLR9* and *LINE1* was associated with serum testosterone.

PBMC *TLR9* and *LINE1* gene expression were both decreased in post pubertal volunteers when analysed in sub-sections 6.2.2. and 6.2.3. When the expression of these genes was analysed by linear regression, for associations with X chromosome number or serum sex hormone, the most parsimonious models did not include oestradiol but only serum testosterone concentration and X chromosome number (Table 6-4). By these models, *LINE1* and *TLR9* PBMC gene expression was found not to differ significantly with X chromosome number, but both associated negatively with serum testosterone concentration (Fig. 6-10).

Model: y=Intercept+B_1*X chromosome number+B_2*Testosterone								
Independent Variables	Dependant variables							
	TLR9 Adjusted r^2=0.078; p=0.024				*LINE1* Adjusted r^2=0.041; p=0.093			
	B	p	95% CI		B	p	95% CI	
X chromosome number (1=0; 2=1)	2.693	0.319	-2.654	8.039	-439.502	0.279	-1243.92	364.922
Testosterone nmol/L	-.385	*0.014	-.691	-.079	-46.599	*0.047	-92.57	-.629

Table 6-4. Linear regression model for PBMC gene expression of TLR9 and LINE1.

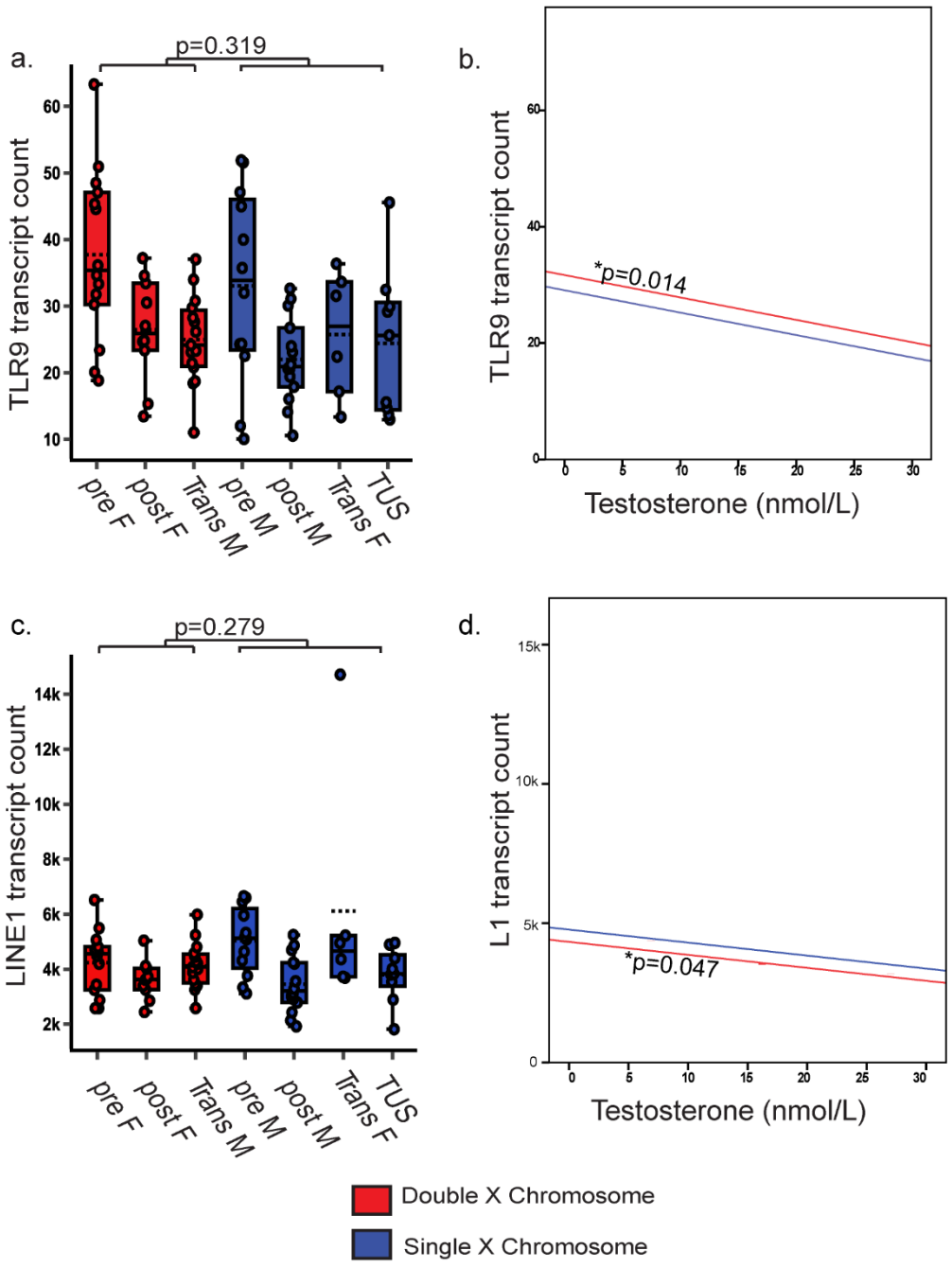


Figure 6-10. PBMC gene expression of LINE1 and TLR9 was decreased in association with serum testosterone. In healthy, TUS and transgender volunteers (n=77, Pre F=14, Post F=10, Trans M=12, Pre M=12, Post M=14, Trans F=6, TUS=9), PBMC *TLR9* and *LINE1* gene expression was measured and analysed by linear regression. a.-b. *TLR9* expression did not differ with X chromosome number(p=0.319) but did associate negatively with serum testosterone (p=0.014). c.*LINE1* expression did not differ with X chromosome number (p=0.279) but did associate negatively with serum testosterone (p=0.047). The lines in b. and d. represent the linear regression equation y=Intercept+B_1*X chromosome number+B_2*Testosterone

6.3.6. The upregulated IFN score associated with serum oestradiol concentration.

The upregulated IFN score was shown above in sub-section 6.2.6 to be higher in post pubertal volunteers. When the model was extended to include transgender and TUS volunteers, there was a significant association with serum oestradiol concentration, with no significant association with X chromosome number or serum testosterone concentration (Table 6-5, Fig. 6-11).

Model: y=Intercept+B_1*X chromosome number+B_2*Testosterone+ B_3*Oestradiol				
Independent Variables	Dependant variable			
	Upregulated IFN score Adjusted r^2=0.247; p=0.015			
	B	p	CI	
X chromosome number (1=0; 2=1)	-522.391	0.189	-1318.90	274.118
Testosterone nmol/L	29.617	0.253	-22.415	81.649
Oestradiol pmol/L	3.511	*0.009	.972	6.049

Table 6-5. Linear regression model testing for associations between upregulated IFN score, X chromosome number and serum sex hormone in healthy, TUS and transgender volunteers.

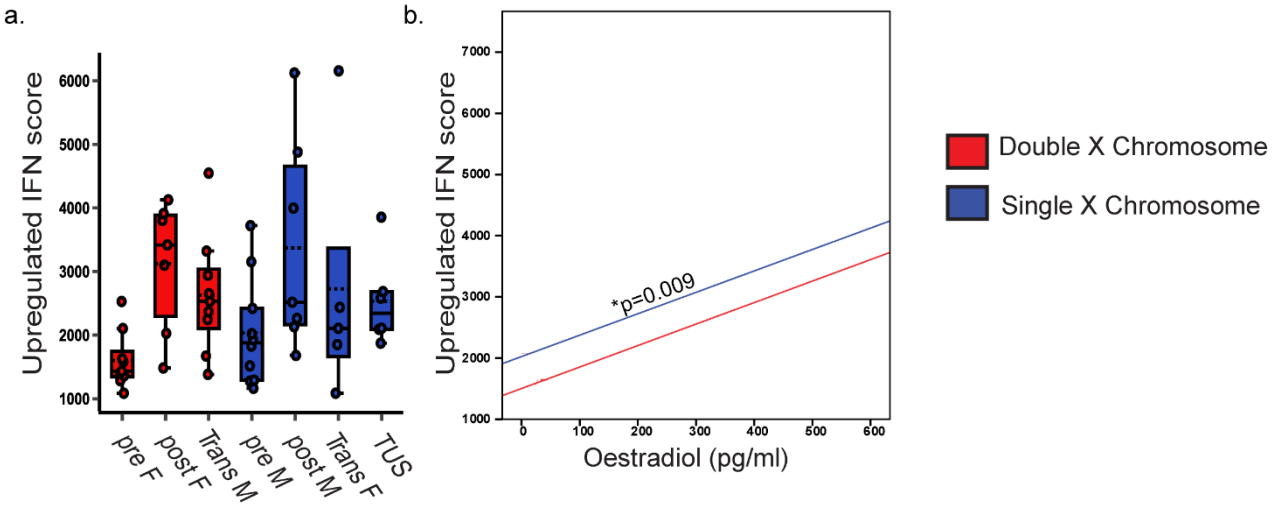


Figure 6-11. Upregulated IFN score associated with serum oestradiol. a. In healthy, TUS and transgender volunteers (n=58, Pre F=9, Post F=7, Trans M=7, Pre M=10, Post M=7, Trans F=5, TUS=6), the upregulated IFN score did not associate with serum testosterone or the number of X chromosomes present but did associate significantly with serum oestradiol (p=0.009) when analysd by linear regression. b. *The lines in b. illustrate the equation y=Intercept+B_1*X chromosome number+B_2*Testosterone+ B_3*Oestradiol for single and double X chromosomes, holding testosterone constant at 0 nmol/L.

6.4. PBMC gene expression in jSLE

Aim: To investigate whether the expression of selected genes in the type 1 IFN pathway differs between healthy young people and those with jSLE.

6.4.1. The IFN score was higher in young people with jSLE and the upregulated IFN score was lower.

In keeping with reported literature(1, 189), it was found that the IFN score was significantly higher in young people with jSLE when compared to healthy, post pubertal volunteers by t-test (p=0.001, mean difference=679.95; 95% CI=293.10, 1066.80) (Fig. 6-12a). A mean IFN score was calculated from the healthy post-pubertal volunteers only. Greater than 2 standard deviations (SD) above the mean was considered IFN positive (IFN+) and less than 2SD above the mean was considered IFN negative (IFN-)(26) (Fig. 6-12b). 14/29 (48%) of young people with jSLE were IFN+, vs 1/39 healthy volunteers.

The upregulated IFN score was lower in young people with jSLE as compared to healthy volunteers, although this did not reach statistical significance (t test: p=0.070; mean difference=1039.74; 95% CI=-91.67, 2171.16) (Fig 6-12c).

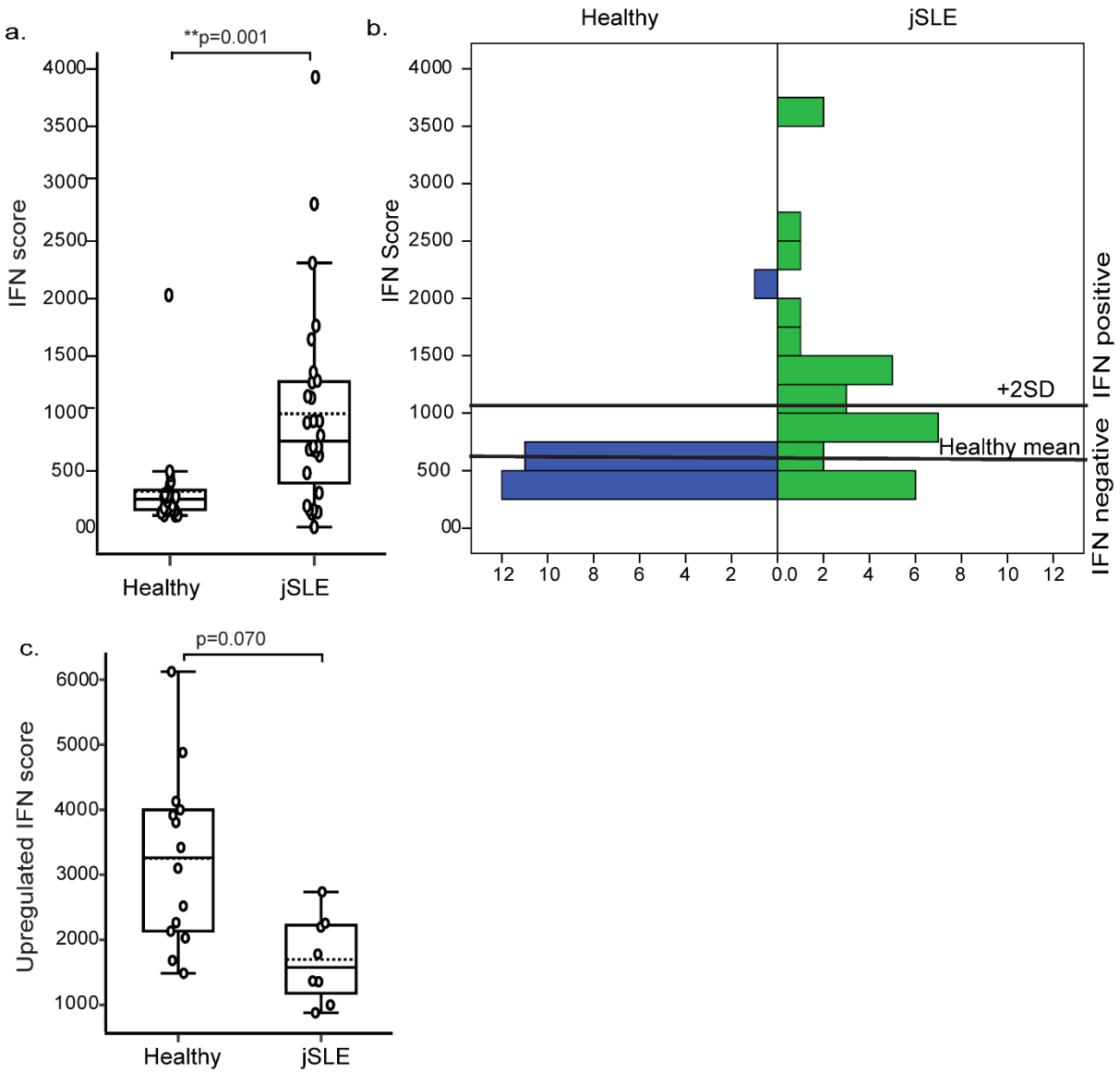


Figure 6-12. The IFN score was higher in young people with jSLE. a. Young people with jSLE had a higher IFN score when compared to healthy volunteers (t test p=0.001, n=79-jSLE=29, healthy=50). b. A normal range was calculated from healthy volunteers. <2SD above the healthy mean=IFN negative and >2SD above the healthy mean =IFN positive. c. Volunteers with jSLE had a lower upregulated IFN score than healthy volunteers (t test: p=0.07, n=23 healthy=14; jSLE=9) although this did not reach statistical significance.

6.4.2. Measuring IFN inducible genes.

jSLE is a disease with an inherent IFN signature. Therefore, to assess whether differences existed in the expression of various genes in the IFN pathway, it needed to be established whether individual genes were IFN inducible. In the healthy, post pubertal volunteers only, PBMC gene expression was measured at baseline (*ex vivo*) and after 20 hours of stimulation with IFNα and significance values were adjusted for multiple testing by Bonferroni method

(Fig. 6-13). It was established that many of the genes measured in the panel were IFN inducible, all except for *DDX41, TLR7, TRAIL* and *IRF5*.

For this reason, when measuring for differences between healthy volunteers and those with jSLE in the expression of genes shown to be IFN inducible, a correction for the IFN score was included to account for the inherent upregulation of ISG in jSLE.

Gene	Corrected p value
*IRF7	0.000000
*MDA5	0.000000
*MX1	0.000000
*RIG-1	0.000000
*MAVS	0.000000
*MCP-1	0.000000
*ISG15	0.000000
*BST2	0.000000
*IFIT-1	0.000001
*L1	0.000003
*TNFR6	0.000024
*TLR9	0.000058
*TROVE2	0.001632
DDX41	0.003687
TLR7	0.018325
TRAIL	0.066654
IRF5	0.067258

Figure 6-13. IFN inducible gene assay. PBMC from healthy volunteers were analysed for gene expression before and after stimulation with IFNα. All p values are adjusted for multiple testing by Bonferroni method. $p<0.003$ indicates that the gene was significantly upregulated by IFNα stimulation and was therefore considered IFN inducible (ISG). Any comparison of ISG in jSLE patients must therefore contain a correction for background IFN score. Values of 0.000000 represent values less than 0.000001.

6.4.3. Among the gene expression of the selected RNA sensors, *RIG-1* was downregulated in jSLE, whereas *TLR7* showed no difference.

There was no difference in the PBMC expression of *TLR7* between healthy volunteers and those with jSLE (B=3.194; p=0.442; CI=-5.080, 11.467) (Table 6-6). The other RNA sensors *MDA5, RIG-1* and signalling protein *MAVS*, were all IFN inducible and were analysed including a correction for IFN score (Table 6-6).After correcting for IFN score, jSLE associated with a decreased PBMC gene expression of *RIG-1* (B=-44.576; p=0.033; 95%CI=-85.286, -3.866) but no significant difference in expression of *MDA5* or *MAVS* was observed (not shown).

It was shown in 6.2.1. that *TLR7* gene expression was higher in post pubertal healthy females as compared to males (p=0.02). In young people with jSLE there was no difference in *TLR7* expression between sexes (t-test, p=0.44). There was a significantly higher expression of *TLR7* in males with jSLE as compared to healthy males (t-test, p=0.045, Fig. 6-14).

Model: y=Intercept+B_1*type+B_2*IFN score												
Independent Variables	Dependant variables											
	TLR7 Adjusted r^2=-0.008, p=0.442				*RIG*-1 Adjusted r2=0.646, p=0.001				*MDA5* Adjusted r2=0.770, p=0.001			
	B	p	95% CI		B	p	95% CI		B	P	95% CI	
Type (0=healthy; 1=jSLE)	3.194	0.442	-5.088	11.467	-44.576	*0.033	-85.286	-3.866	19.616	0.128	-5.852	45.085
IFN score					0.127	0.001	0.100	0.153	0.92	0.001	0.075	0.108

Table 6-6. Linear regression models testing for differences in RNA sensing pathway gene expression between healthy volunteers and those with jSLE. (n=79) As *TLR7* was not IFN inducible, there was no correction for IFN score as there was in *RIG-1* and *MDA5*. After correcting for IFN score, it was found that RIG-1 was downregulated in jSLE (p=0.033). *p<0.05

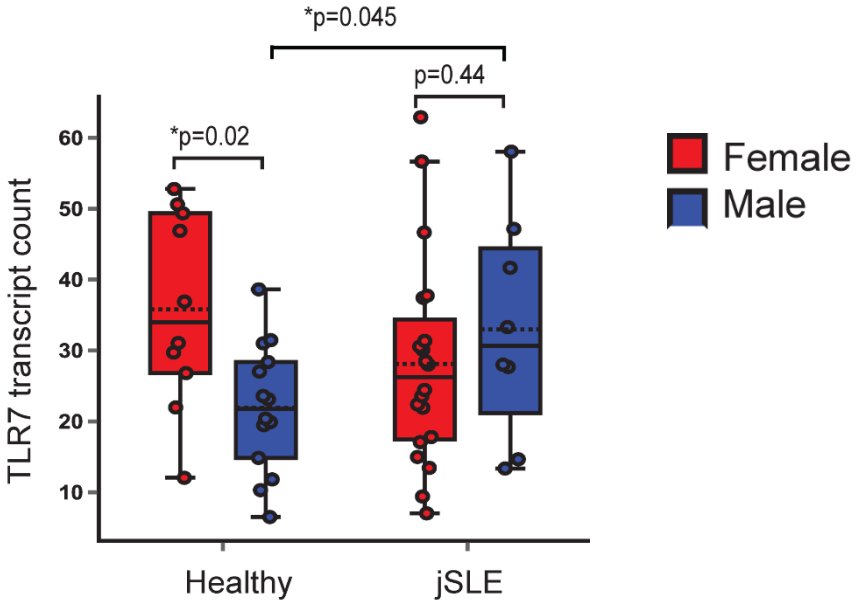


Figure 6-14. PBMC TLR7 gene expression sex difference was lost in jSLE. The gene expression of *TLR7* was significantly higher in healthy, post pubertal females as compared to males (p=0.02) when analysed by t-test. This difference was lost in jSLE (p=0.44), and males with jSLE expressed significantly more *TLR7* gene in PBMC than healthy males (p=0.045) (Healthy M=14, Healthy F=10, jSLE F =20, jSLE M=9).

6.4.4. The expression of *TLR9* was decreased in jSLE, after controlling for IFN score.

There was a relatively decreased gene expression of *TLR9* in jSLE (B= -6.076; p=0.038; 95% CI=-11.801, -0.352) after controlling for IFN score (Table 6-7, Fig. 6-15). There was a relatively decreased PBMC gene expression of *DDX41* in jSLE after controlling for IFN score, but this did not reach statistical significance (B=-13.051; p=0.093; 95% CI=-29.314, 2.311).

In addition, there was no significant difference in expression of *IRF5* (B=-11.591; p= 0.739; 95% CI= -81.031, 57.850) and *IRF7* (controlling for IFN score) (B=65.726; p= 0.245; 95% CI=-46.538, 177.989) in jSLE as compared to healthy volunteers (data not shown).

Model: y=Intercept+B_1*type+B_2*IFN score								
Independent Variables	Dependant variables							
	TLR9 Adjusted r^2=0.07, p=0.061				DDX*41* Adjusted r2=0.209, p=0.001			
	B	p	95% CI		B	p	95% CI	
Type (0=healthy; 1=jSLE)	-6.076	*0.038	-11.801	-0.352	-13.051	0.093	-29.314	2.311
IFN score	0.004	0.052	0.00	0.007	0.020	**0.001	0.010	0.031

Table 6-7. Linear regression models testing for differences in *TLR9* and *DDX41* PBMC gene expression between healthy volunteers and those with jSLE. *p<0.05

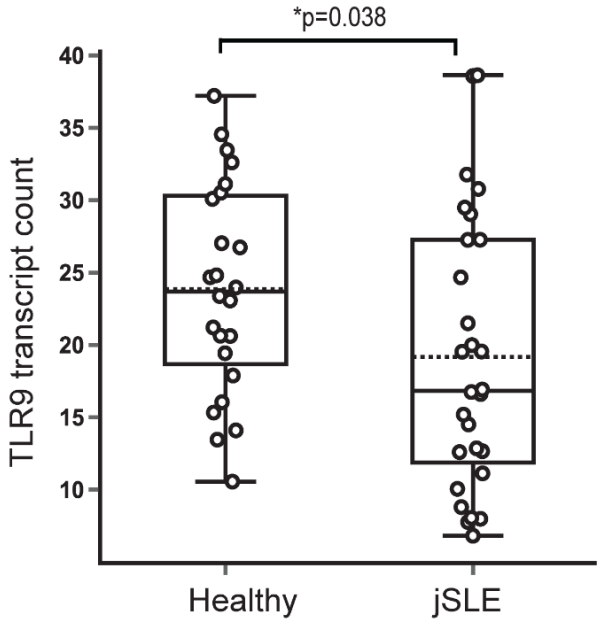


Figure 6-15. TLR9 expression is relatively decreased in jSLE. After controlling for IFN score by linear regression, it was shown that *TLR9* expression was relatively decreased in jSLE (p=0.038) (jSLE=29, healthy=24)

6.4.5. The expression of *LINE1* trended towards being increased in jSLE, after controlling for IFN score.

The PBMC gene expression of *LINE1* was higher in jSLE (B=621.642; p=0.093; 95% CI=-107.270, 1350.554) after correcting for IFN score, although this did not reach statistical significance (Fig. 6-16). There was no difference in PBMC *TROVE-2* gene expression between jSLE and healthy volunteers, controlling for IFN score (B=4.669, p=0.794; 95% CI=-31.119, 40.457).

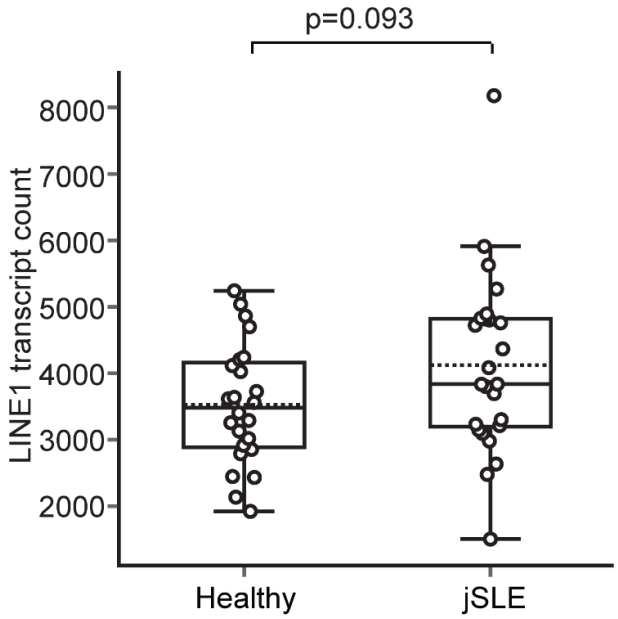


Figure 6-16. LINE1 gene expression trended towards an increase in jSLE after correcting for IFN score. After correcting for IFN score by linear regression, there was a non-significant trend for *LINE*1 expression to be higher in jSLE as compared to healthy volunteers (p=0.093) (jSLE=29, healthy=24).

6.4.6. There was no difference in gene expression of *TRAIL* or *TNFR6* in jSLE.

There was no difference in the expression of apoptosis related genes *TRAIL* (B=45.689; p= 0.588; 95% CI= -122.643, 214.022) or *TNFR6* (correcting for IFN score-B= 24.545; p=0.147; 95% CI=-8.929, 58.018) in jSLE.

6.5. Discussion

In this chapter, it was seen that females in general, had a higher gene expression of the intracytoplasmic RNA sensors, *RIG-1* and *MDA5*. Post-pubertal females specifically had a higher gene expression of *TLR7*, an endosomal RNA sensor. In further analysis, it was shown that at least RIG-*1* gene expression was significantly related to X chromosome number, and that none of the genes encoding these RNA sensors associated with serum sex hormone concentrations.

Females are known to have an increased response against RNA viruses such as HIV (43)and influenza (268), as well as an increased risk of developing jSLE(4). It is shown here that females expressed more of these RNA sensors than males, which may contribute to our understanding of this sex difference.

Interestingly, *TLR7* was the only gene out of these RNA sensors that is located on the X chromosome. Recent work has shown that *TLR7* may escape X inactivation in a significant proportion of B cells, monocytes and pDC in healthy women and men with Klinefelter's syndrome (XXY)(127). In the same study, B cells that were bi-allelic for *TLR7*, after stimulation

with a TLR7 agonist, showed a preferential enrichment for plasma cells and increased Ig class switching. This has led researchers to speculate as to whether *TLR7* may escape X inactivation in females, which predisposes them to develop SLE. It is therefore intriguing that, in the healthy young volunteers, *TLR7* was significantly increased in post pubertal females, but did not relate statistically to either X chromosome number, serum testosterone or oestradiol. There are other puberty associated hormones, which were not tested here, such as LH or FSH, and many other non-hormonal parameters that may associate with this rise in *TLR7* expression in females after puberty. It would be interesting to investigate in the future, whether the increase in *TLR7* gene expression in post pubertal females relates to an increase in the bi-allelic expression of *TLR7* on the X chromosome over puberty. This may be due to epigenetic mechanisms that associate with puberty.

In this study, no difference was seen in the PBMC gene expression of *TLR7* between healthy volunteers and those with jSLE. Gene expression of *TLR7* in B cells has been reported to be increased in jSLE(237). Whole blood gene expression of *TLR7* and *TLR9* have been reported to be increased in adult patients with active SLE(239, 269). Among this low disease activity group however, there was no significant difference in *TLR7* expression.

The sex difference in *TLR7* gene expression that was seen in healthy young people was not seen in jSLE and there was a significantly higher expression of *TLR7* in males with jSLE as compared to healthy males. The BXSB lupus model is a recombinant inbred mouse strain produced from a cross of C57BL/6J (B6) and SB/Le mice(229, 230). Male BXSB mice develop severe lupus-like autoimmunity, which depends on the presence of the y-linked autoimmune accelerator (yaa) region, on the background of other autoimmune susceptibility genes. It has been demonstrated that yaa contains a translocation of *TLR7* from the X chromosome onto the Y chromosome, resulting in a 2-fold increase in expression of *TLR7* which is sufficient to induce the phenotype in males. Another study showed that human adult males with a *TLR7* SNP, associated with an increased expression of *TLR7*, had a higher risk of developing SLE as compared to females with the same mutation(270). Altogether, these data all seem to indicate that an increased *TLR7* expression associates more with SLE in males, which is perplexing, given that it is coded for on the X chromosome and would provide a logical explanation for the female predominance of SLE. Indeed, the finding that healthy females had an increased expression of *TLR7* after puberty does suggest that it may relate to the increased prevalence of jSLE in females upon sexual maturity, but *TLR7* gene expression was not directly increased in females with jSLE. Perhaps, the increased expression of *TLR*7 in healthy females after puberty contributes to their increased risk of jSLE, whereas it may be more likely to pathogenic if raised in males.

Conversely, it was seen that the PBMC *TLR9* gene expression was decreased in post pubertal volunteers. Various murine lupus models have demonstrated that *TLR9* expression may be protective against disease development, whereas *TLR7* gene expression mediated inflammation, IFNα production and disease progression (234, 235, 271, 272). Importantly, despite no baseline difference in IFN score, the upregulated IFN score was significantly higher in the post pubertal volunteers, which associated significantly with serum oestradiol, after correcting for X chromosome number and serum testosterone. This too may predispose females with a higher oestradiol to develop jSLE after puberty, with a more rigorous gene response to IFN stimulation.

Perhaps, the combination of a pubertal decrease in *TLR9* gene expression, regardless of sex, along with an increased ability to upregulate ISG after puberty, linked specifically to oestradiol, may contribute to the increased risk of developing jSLE after puberty. Additionally, the specific increase in *TLR7* expression after puberty in healthy females may account for at least some of the increased susceptibility seen in young women.

Interestingly, in jSLE, as expected, the baseline IFN score was significantly higher than healthy volunteers, but the upregulated IFN score trended towards being lower than healthy volunteers. This suggests perhaps that PBMC in jSLE may have a level of exhaustion or relative downregulation in their ability to upregulate ISG in response to further IFN challenge.

A limitation of these data is that the gene expression is not specific to a certain cell type but is from PBMC. It was not possible to get enough PBMC to separate out specific cells types (e.g. pDC) and assess their individual gene expression, which would be more informative.

When studying gene expression in jSLE, it is difficult to tease out true differences, as many genes that may be of interest, are inherently over-expressed simply because they are ISG. In this study, the IFN score was used to control for the effect of background ISG upregulation when investigating the pattern of expression of individual ISG of interest in jSLE. This has not been done previously in other studies, and probably represents more accurate results. Indeed, genes such as *TLR9* and *RIG-1*, which are ISG and have been reported to be upregulated in jSLE were relatively downregulated when the IFN score was controlled for.

The ERE, *LINE1* is known to be abundant in the human genome, compromising 17% of human DNA (218). Indeed, in this study, the expression of *LINE1* gene was higher than any other gene included in the panel. In the absence of virus, it has been proposed that ERE may act as an endogenous source of nucleic acid substrate in SLE(219). It has been shown that *LINE1* RNA transfection is capable of inducing TLR7 mediated type 1 IFN production (220). In addition, the

expression of ERE has been shown to be enriched in patients with SLE and is related to disease activity, although it is unclear whether this is due to primary disease or is due to an increased IFN signature, as *LINE1* is known to be IFN inducible(221). In these data, it was shown that LINE1 gene expression trended towards being higher in jSLE, after correcting for IFN score. Even though this didn't reach statistical significance, *LINE1* was the only IFN inducible gene that showed a relative increase when correcting for ISG. This is important, as due to its abundance, *LINE1* activity may be a logical target for future investigation for use in therapy of jSLE.

6.6. Conclusion

In this chapter, it was shown that in females, there was a higher PBMC gene expression of the RNA sensors, *RIG-1* and *MDA5* in general, and an increased expression of *TLR7* in post pubertal females specifically. PBMC gene expression of *TLR9* and *LINE1* was lower in post pubertal volunteers with no sex difference observed, whereas the upregulated IFN score was significantly enhanced in post pubertal volunteers. There was no overall difference in PBMC *TLR7* gene expression between healthy volunteers and those with jSLE, but males with jSLE did have a significantly higher *TLR7* gene expression than healthy males. After correcting for the IFN score, PBMC gene expression of *TLR9* was relatively decreased in jSLE, whereas *LINE1* gene expression trended towards an increase.

It was next investigated whether stratifying volunteers with jSLE as IFN positive or negative, revealed any further differences which were not evident when studying jSLE as a uniform group.

Chapter 7: Stratifying jSLE by IFN score

It was reported in chapter 6 that 48% of young people with jSLE were IFN+. It was therefore investigated in a post hoc analysis, whether stratifying jSLE by IFN score showed further differences in immune phenotype, pDC function and PBMC gene expression.

7.1. Clinical differences between IFN + and IFN – jSLE.

Aim: To investigate whether clinical differences exist between young people with IFN+ and IFN- jSLE.

Sub aims:

i. To assess whether demographic differences (sex and ethnicity) exist between young people with IFN+ and IFN- jSLE.
ii. To assess whether differences exist in disease activity, auto-antibody presence, drug therapy and organ involvement between young people with IFN+ and IFN- jSLE.

7.1.1. Demographics

7.1.1.2. Sex

Fourteen out of twenty-nine (48%) patients with jSLE were IFN + (>2SD above the healthy mean). Nine out of eleven (45%) females were IFN+ vs five out of nine (55%) males. There was no significant sex difference in the percentage IFN+ when assessed by Chi squared test (p=0.599, Table 7-1.). The odds of being IFN+ were not significantly different between sexes when analysed by logistic regression (ExpB=1.528; p=0.60; 95% CI=0.314, 7.437).

		IFN score		Total
		Negative (<2SD)	Positive (>2SD)	
Sex	Female	11	9	20
	Male	4	5	9
Total		15	14	29

Table 7-1. There was no sex difference in the number of jSLE patients who were IFN+ or -.

7.1.1.3. Ethnicity

There was no significant difference in the likelihood of having a positive IFN score between ethnicities when analysed by logistic regression (Table 7-2). Although not significant, probably due to small numbers (only 6 black patients in total) it should be noted that black patients had a 6-fold increase in the likelihood of being IFN+ than white patients (ExpB =6; p=0.152; 95% CI=0.516, 69.754).

		IFN score		Total	Significance of B (p value)	Exp (B)	95% CI Exp (B)	
		Negative (<2SD)	Positive (>2SD)					
Ethnic groups	White*	4	3	7				
	South Asian	3	2	5	0.839	0.800	0.093	6.848
	Mixed	1	0	1	1.000	0.000	0.000	
	Black	1	5	6	0.152	6.000	0.516	69.754
	East Asian	3	2	5	0.839	0.800	0.093	6.848
	Other **	2	2	4				
Total		14	14	28				

Table 7-2. There were no significant differences in likelihood of being jSLE IFN+ or – based on ethnicity*White was reference category in model so no coefficient displayed. ** Other not included in logistic regression model as only 2 values.

7.1.2. Disease activity

There were no significant differences in prednisone dose, SLEDAI, or clinical markers of disease activity (ESR, CRP, DsDNA, WCC or serum complement components, C3 or C4) between young people with jSLE who were IFN+ or IFN- when analysed by Mann Whitney U test.

	IFN- (n=15)		IFN+ (n=14)		
	Median	Range	Median	Range	Mann Whitney u test p-value
Prednisone (mg/day)	0.0	0-10	4.0	0-15	0.310
SLEDAI	2	0-10	3	0-24	0.635
dsDNA (IU/ml)	31	0-546	48	48-2827	0.905
ESR (mm/hr)	8	2-29	9	2-47	0.210
CRP (mg/L)	0.60	0.6-3.1	0.8	0.6-3.7	0.880
White cell count ($x10^9$/ml)	4.90	3.5-10.7	5.16	1.07-7.28	0.830
C3 (g/L)	1.07	0.71-1.44	0.98	0.33-1.7	0.804
C4 (g/L)	0.14	0.06-0.29	0.185	0.0-0.43	0.301

Table 7-3. There were no significant differences in prednisone dose or clinical markers of disease activity between patients with jSLE who were IFN+ or IFN-.

7.1.3. Auto-antibodies

After correcting for all the measured auto-antibodies, if a patient had auto-antibodies towards RNP, the IFN score was on average 1010.94 counts higher than if they did not (p=0.044, 95% CI = 27.84, 1993.99) (Table 7-4, Fig. 7-1). By t-test (i.e. without correcting for the presence of other auto-antibodies), this translated to a mean difference in titre of 859 (p=0.018; 95% CI=162.57, 1555.94). When classifying IFN score into binary (+vs-) based on 2SD from the healthy mean, the presence of auto-antibodies towards RNP increased the odds of being IFN+ nearly 5 times (Exp B=4.875; p=0.089; 95% CI=0.785, 30.29) although this effect did not reach statistical significance.

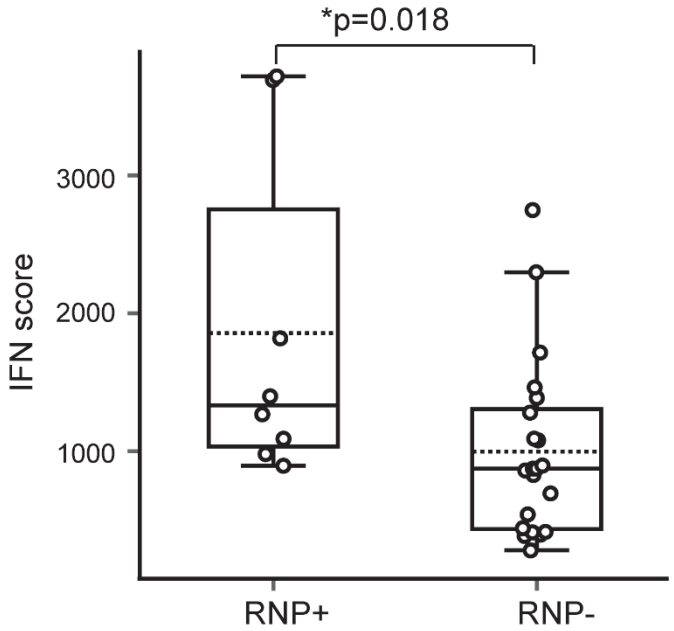


Figure 7-1. IFN score was significantly higher if antibodies towards RNP were present. When analysed by t-test there was a significantly higher IFN score in the group that were RNP positive (p=0.018).

Antibody (positive vs negative)	B	p	95% CI	
RNP	1010.914	*0.044	27.841	1993.988
Ro	216.805	0.581	-585.323	1018.933
La	689.093	0.194	-376.736	1754.923
Sm	81.334	0.876	-986.268	1148.936
dsDNA	-131.130	0.765	-1027.691	765.431

Table 7-4. RNP associated significantly with IFN score after correcting for the presence of other auto-antibodies. A linear regression model was fitted to investigate for associations of the presence of auto-antibodies to IFN score. Only autoantibodies towards RNP associated significantly with IFN score after controlling for the presence of the other measured autoantibodies.

7.1.4. Organ involvement

Current or previous organ involvement in young people with jSLE was recorded. Only two patients had either neurological or cardiac disease respectively, and only two patients did not have skin disease. Therefore skin, neurological and cardiac involvement were excluded from the analysis as they skewed results.

When assessed by linear regression, the presence of renal, haematological or joint disease did not associate significantly with the IFN score (Table 7-5).

Organ system	Involved?	Count		IFN Score			B	p
		IFN+	IFN-	Mean	95% CI			
Skin	no	2.00	0.00	411.48	-1244.5	2067.48		
	yes	12.00	14.00	1330.74	969.68	1691.79		
Haematological	no	10.00	6.00	1041.21	746.30	1336.12	583.32	0.130
	yes	5.00	8.00	1473.73	767.85	2179.61		
Neurological	no	13.00	14.00	1296.55	942.46	1650.64		
	yes	2.00	0.00	405.48	281.91	529.04		
Renal	no	9.00	12.00	1373.87	925.34	1822.41	-390.55	0.351
	yes	6.00	2.00	870.81	493.59	1248.03		
Arthritis	no	7.00	4.00	1227.35	513.76	1940.94	164.24	0.656
	yes	5.00	9.00	1371.37	886.74	1856.00		
Cardiac	no	13.00	11.00	1184.08	849.12	1519.03		
	yes	2.00	3.00	1479.99	-217.44	3177.43		

Table 7-5. Linear regression showed that IFN score did not associate with the presence of haematological, renal or joint disease.

7.1.5. Drug therapy

Only one patient was treatment naïve. This patient was newly diagnosed and had a high IFN score and a high disease activity. This individual was found to be highly influential in analysis of associations between drugs and IFN score so was excluded from these analyses (Fig. 7-2). No significant associations were seen between IFN score, mycophenylate dosage (B=0.255; p=0.453; 95% CI=-0.445, 0.96), azathioprine dosage (B=5.797; p=0.254; 95% CI=-4.57, 16.16) or hydroxychloroquine dosage (B=-1.669; p=0.53; 95% CI=-7.16, 3.82, Fig. 7-2.) when analysed by multiple linear regression.

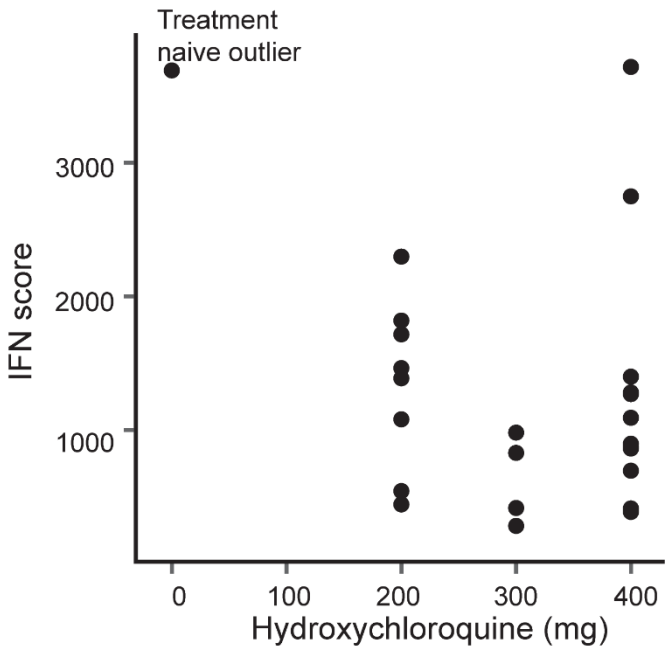


Figure 7-2. HCQ dose did not associate with IFN score. After an influential treatment naïve outlier was excluded, it was found that HCQ did not associate significantly with IFN score (p=0.53) by linear regression.

7.2. Differences in immune cell phenotype in jSLE stratified by IFN score.

It was previously shown in chapter 4 that the percentage of BDCA2+ pDC and CD11c+ cDC in PBMC was significantly lower in young people with jSLE, while there was a higher percentage of CD4+ and CD8+ T cells in jSLE. In addition, B cells and pDC from volunteers with jSLE expressed more of the IFN inducible surface marker, tetherin, when compared to healthy volunteers. It was investigated whether stratifying patients with jSLE showed any further differences in PBMC subset percentage or tetherin expression.

Aim: To investigate whether differences exist in immune cell phenotype when jSLE is stratified by IFN score.

Sub-Aims:

i. To investigate whether differences exist in the percentage of each PBMC cell subtype when jSLE is stratified by IFN score.
ii. To investigate whether differences exist in the expression of tetherin by pDC, CD4+ T cells, CD8+ T cells and B cells, when jSLE is stratified by IFN score.

7.2.1. B cell depletion did not associate with IFN score.

Four of the patients with jSLE were B cell depleted (<2% of PBMC). There was no association between the percentage of CD19+ B cells in PBMC and the IFN score (B=-1.424; p=0.956; CI=-54.66, 51.82, Fig. 7-3). Due to their influence on the relative expression of other cell types however, these volunteers were excluded from further analysis of cell subtype percentage.

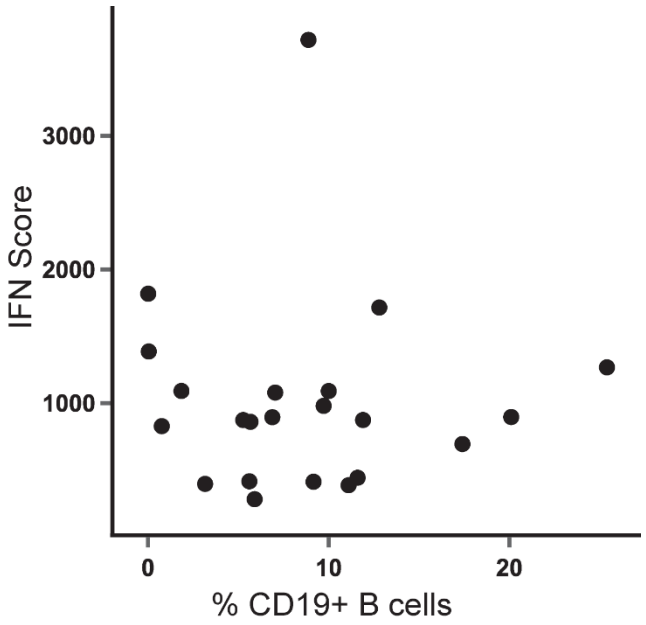


Figure 7-3. IFN score did not associate with the percentage of CD19+ B cells in PBMC. Analysed by linear regression.

7.2.2. PBMC subtypes when jSLE was stratified by IFN score.

The percentage of individual PBMC cell types were analysed by ANOVA with post hoc testing and Bonferroni adjustment for multiple correction. There was a decrease in the percentage of BDCA2+ pDC (ANOVA p=0.072) between IFN+ jSLE, IFN- jSLE and healthy volunteers, although this did not reach statistical significance (Fig 7-4a). There were no overall significant differences in the percentage of CD19+ B cells (ANOVA p=0.323); CD4+ T cells (ANOVA p=0.507); CD8+ T cells (ANOVA p=0.139) and CD14+ monocytes (ANOVA p=0.298) between groups (Fig. 7-4b,c,d,f). There was a significant difference in the percentage of CD11c+ cDC between healthy volunteers and those with IFN+ jSLE (p=0.035) (Fig 7-4e).

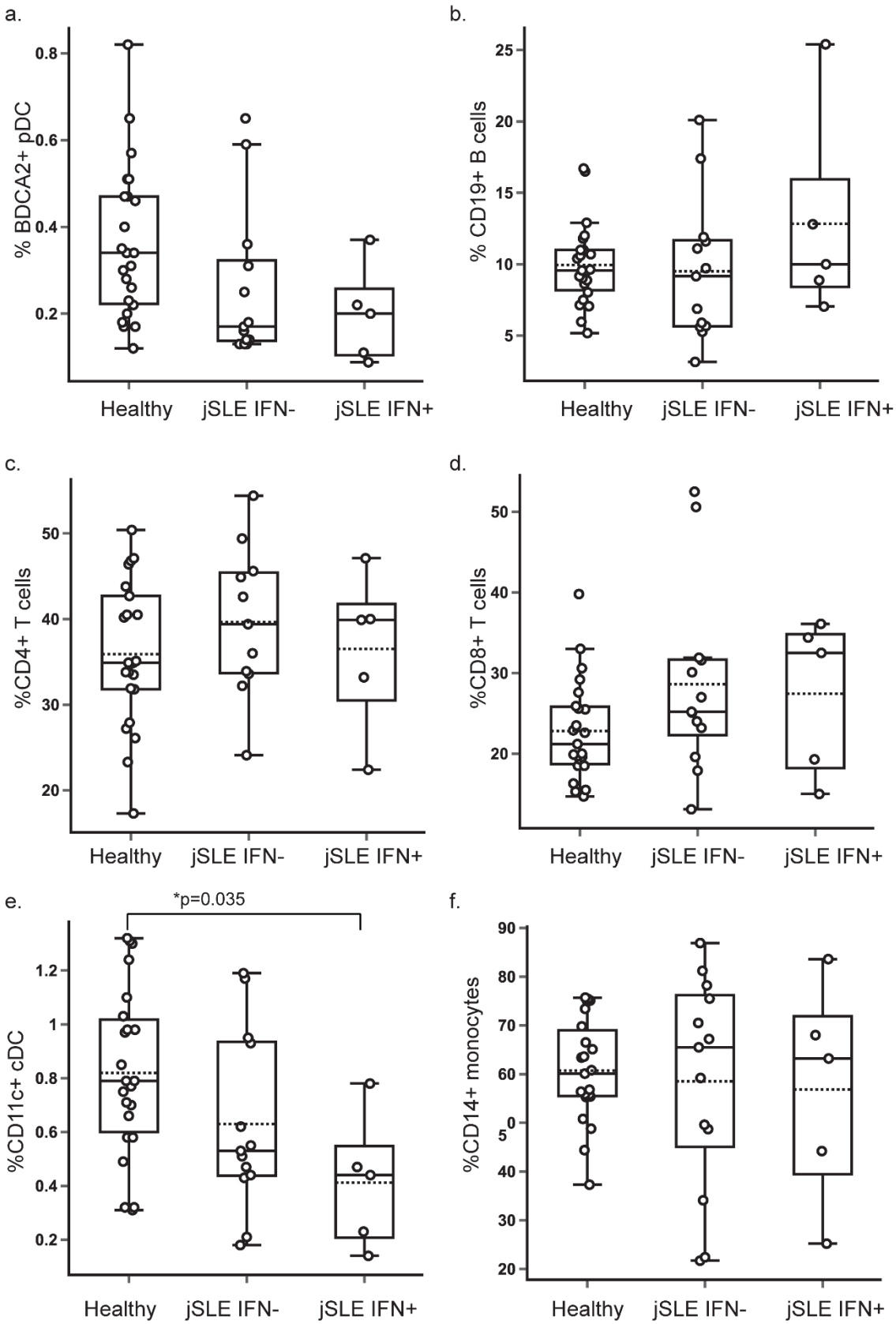


Figure 7-4. PBMC cell subtype stratified by IFN score. There was no significant difference in the percentage of a. pDC, b. CD19+ B cells, c. CD4 T cells, d. CD8 T cells or f. CD14+ monocytes when stratified by IFN score by ANOVA with Bonferroni correction . e. There was a significantly higher %CD11c+ DC between healthy volunteers and IFN- jSLE (adjusted post hoc p=0.035). (Healthy=22; jSLE IFN-=14, IFN+=8).

7.2.3. Tetherin expression when jSLE was stratified by IFN score.

Surface tetherin expression was measured by flow cytometry on all cell types in the lymphocyte compartment and compared by ANOVA with post hoc analysis and Bonferroni correction for multiple testing (analysis summarised Table 7-6, Fig. 7-5).

Tetherin expression was significantly higher in IFN+ vs IFN- jSLE in CD4+ T cells (p=0.006) and CD8+ T cells (p=0.007) (Fig 7-5a, b.). Tetherin expression was higher in IFN+ jSLE than healthy controls in CD4+ T cells (p=0.077); CD8+ T cells (p= 0.012); pDC (p=0.004) and B cells (p=0.034).

Cell type	Post hoc test	P value (corrected)	Mean difference	95% CI	
CD4+ T cell tetherin MFI	healthy vs IFN+	0.077	33.7	4.624	122.653
	IFN- vs IFN+	*0.006	92.714	22.968	162.46
CD8+ T cell tetherin MFI	healthy vs IFN+	*0.012	143.663	26.076	261.250
	IFN- vs IFN+	*0.007	164.536	37.569	291.502
pDC tetherin MFI	healthy vs IFN+	**0.004	284.326	80.089	488.562
	IFN- vs IFN+	0.121	187.071	33.456	407.599
B cell tetherin MFI	healthy vs IFN+	*0.034	154.16	9.02	299.3

Table 7-6. PBMC cell type tetherin expression. PBMC cell type tetherin expression was measured and compared by ANOVA with Bonferroni post hoc correction.

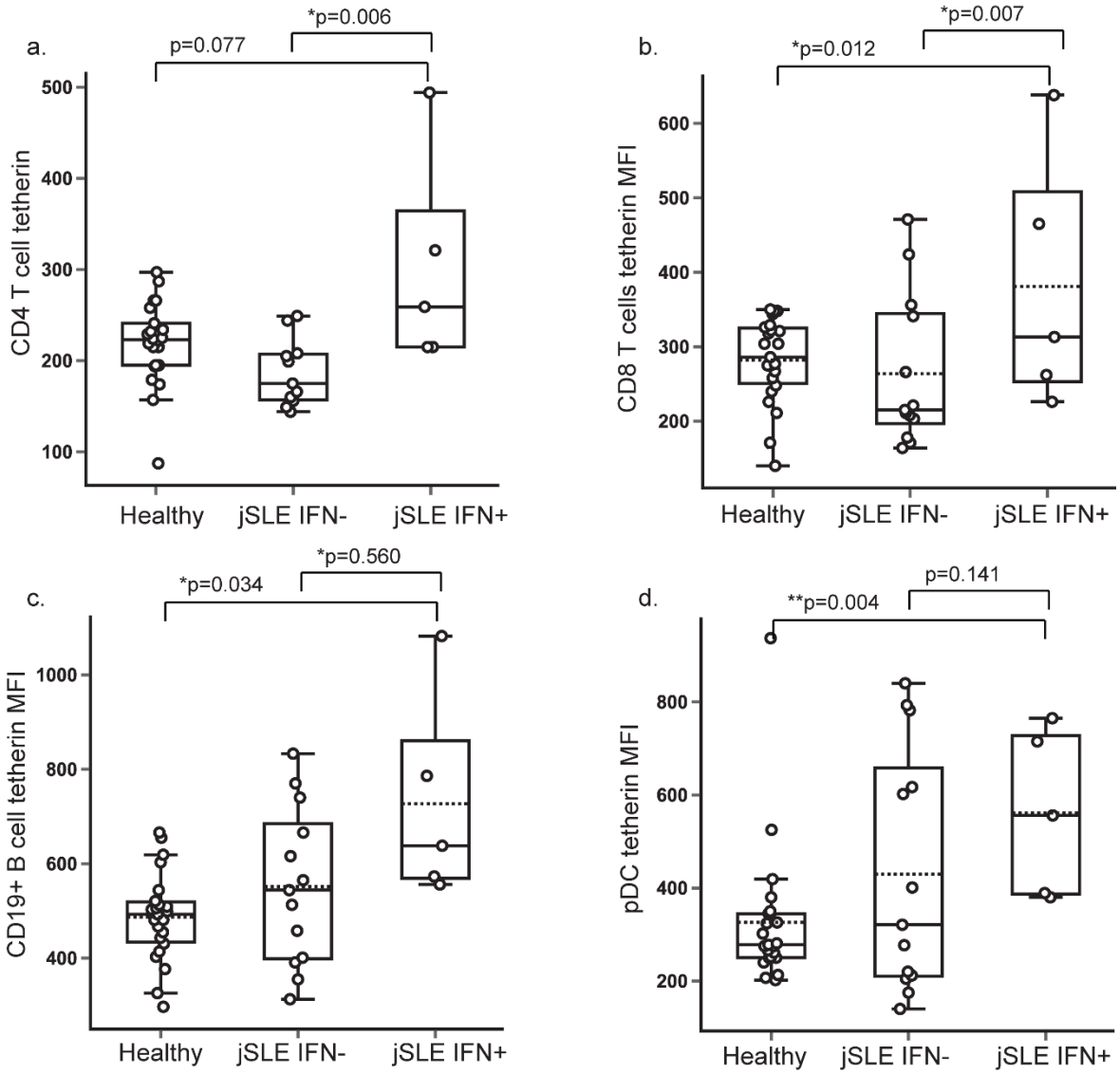


Figure 7-5. CD4+ T cell tetherin surface expression was higher in IFN+ jSLE. a. CD4+ T cell tetherin was higher in IFN+ vs IFN- jSLE (p=0.006). b.CD8+ T cell tetherin was higher in IFN+ vs IFN- jSLE (p=0.007). c-d. B cell (p=0.560) or pDC (p=0.141) tetherin expression was not significantly different between IFN+ vs IFN- jSLE. ANOVA was performed and p values are all corrected by Bonferroni method. (Healthy=22; jSLE IFN-=14, IFN+=8).

7.2.4. CD4+ T cell tetherin expression correlated strongly with IFN score.

After noting that CD4+ T cell expression was significantly higher in IFN+ vs IFN- jSLE, it was investigated how well surface tetherin expression on CD4+ T cells correlated with IFN score in volunteers with jSLE only. CD4+ T cell tetherin expression correlated strongly with IFN score in jSLE (Pearson R^2=0.878; p=0.001, Fig. 7-6.). There was one jSLE patient with a high IFN score and CD4+ T cell tetherin expression. When this patient was excluded, there was still a

significant correlation (Pearson R^2=0.634; p=0.005), implying that this individual was not influential.

There were significant correlations between IFN score and surface tetherin expression on CD8+ T cells (Pearson R^2=0.617; p=0.002); pDC (Pearson R^2=0.423; p=0.050) and B cells (Pearson R^2=0.601; p=0.003) although none reached the same strength of correlation as CD4+ T cell tetherin expression.

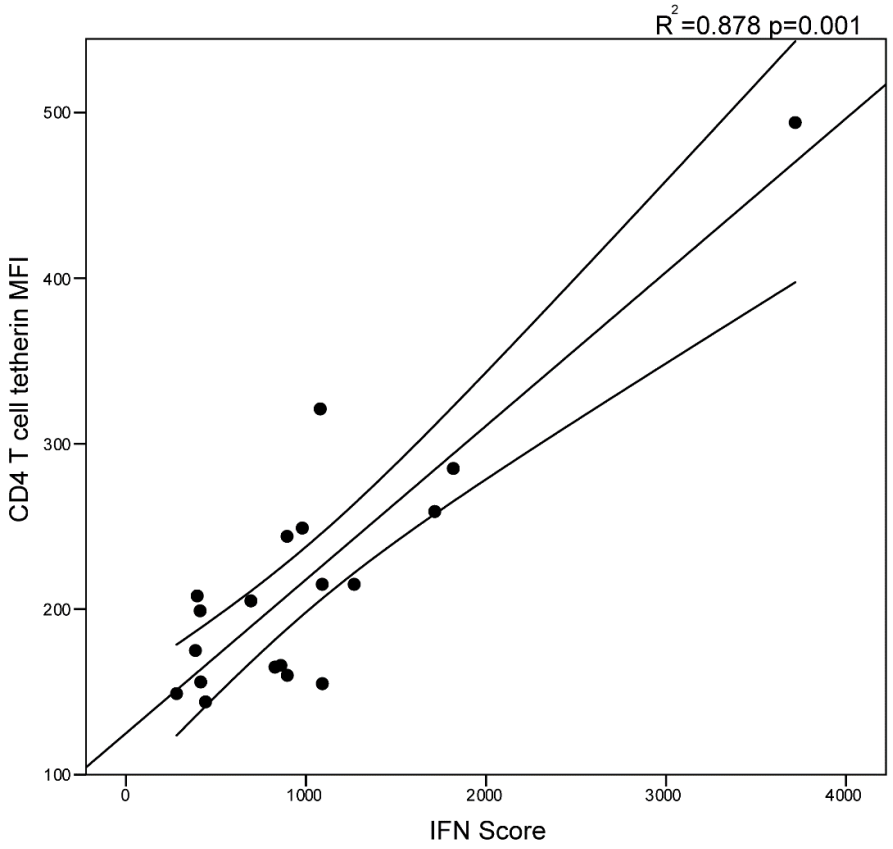


Figure 7-6. There was a strong correlation between CD4+ T cell tetherin expression and PBMC IFN score in jSLE. n=20

7.3. TLR induced pDC IFNα production in jSLE stratified by IFN score.

In chapter 5, it was seen that there was no difference in the TLR7 induced production of IFNα between volunteers with jSLE and healthy volunteers. It was investigated whether differences existed when jSLE was stratified according to IFN score.

7.3.1. There were no differences in TLR7 induced pDC IFNα production when jSLE was stratified by IFN score.

There were no significant differences in the percentage of pDC producing IFNα after R848 stimulation between healthy volunteers and those with jSLE (either IFN+ or IFN-) when analysed by ANOVA with post hoc testing and Bonferroni adjustment (Fig 7-7a). There was a significantly lower percentage of pDC producing IFNα after CpG stimulation in IFN+ (p=0.019;

mean difference=15.677; 95% CI=1.410, 19.434) and IFN- (p=0.002; mean difference=10.422; 95% CI= 1.41, 19.434) jSLE when compared to healthy volunteers (Fig. 7-7b). There was no significant difference in the percentage of pDC producing IFNα between IFN+ and IFN- jSLE however (p=0.709; mean difference=5.225; 95% CI=-5.67, 16.187).

Patients with jSLE are often lymphopenic and volunteers with jSLE frequently had fewer PBMC per ml of blood. For this reason, only 3 volunteers with jSLE who were IFN+ had sufficient cells to stimulate with CpG or R848 and measure the PBMC production of cytokine into supernatant. For this reason, these data were not analysed in the post hoc stratification of jSLE by IFN score.

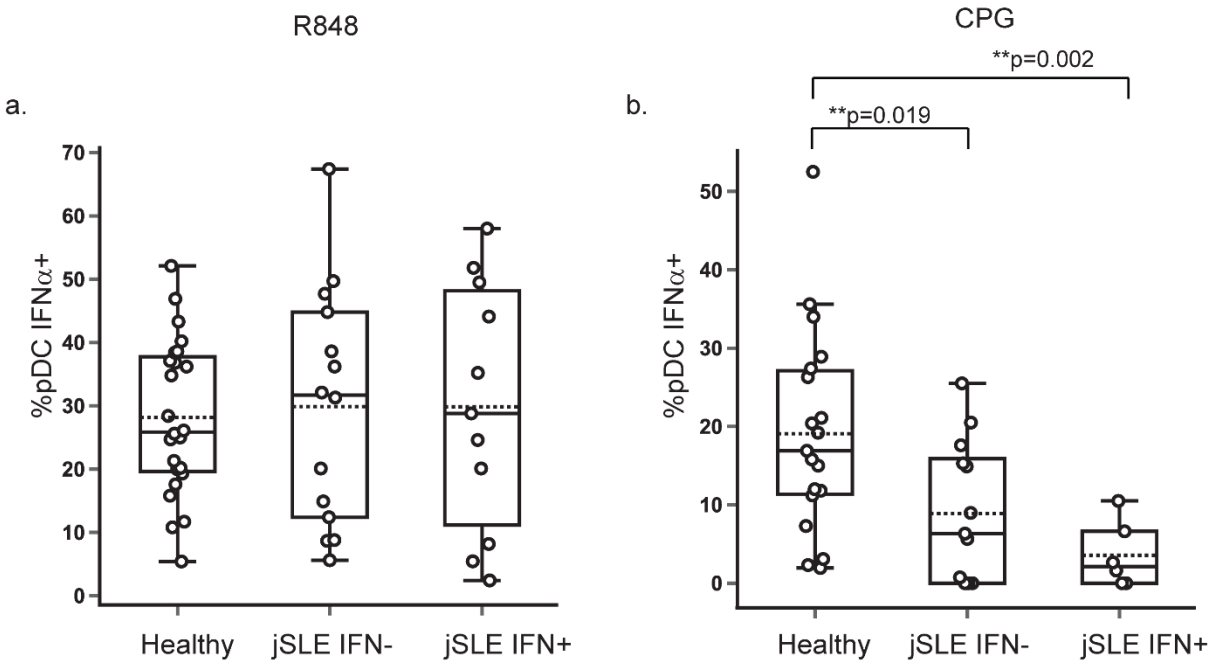


Figure 7-7. IFN+ jSLE volunteers have significantly less pDC producing IFNα after CpG stimulation. a. There were no significant differences in the percentage of pDC producing IFNα between healthy volunteers and IFN+ or IFN- jSLE after R848 stimulation when analysed by ANOVA with Bonferroni correction. b. After CpG stimulation there was significantly lower percentage of pDC producing IFNα in IFN- (p=0.019) and IFN+ (p=0.002) jSLE when compare to healthy volunteers(Healthy=24; jSLE IFN-=15, IFN+=14).

7.4. Gene expression in jSLE stratified by IFN score.

In chapter 5 it was shown that there was no significant difference in PBMC gene expression of *TLR7* in jSLE as compared to healthy volunteers. After correcting for IFN score, there was a relative decrease in *TLR9* and *RIG-1* gene expression and a relative increase in *LINE1* gene

expression in jSLE. A post hoc analysis was performed to assess for differences in gene expression when jSLE was stratified by IFN score.

7.4.1. *TLR7* gene expression was significantly higher in IFN+ jSLE.

TLR7 had been confirmed to not be significantly IFN inducible in 6.4.2. and was therefore analysed by ANOVA with Bonferroni corrected post hoc analysis, without correcting for IFN score.

There was a significantly higher PBMC gene expression of *TLR7* in IFN+ jSLE than healthy volunteers (p=0.022; mean difference=12.615; 95% CI=1.4-23.773; Figure 7-8). Patients with jSLE who were IFN+ had a significantly higher PBMC *TLR7* gene expression than IFN- jSLE (p=0.002; mean difference=18.214; 95% CI=5.883, 30.54).

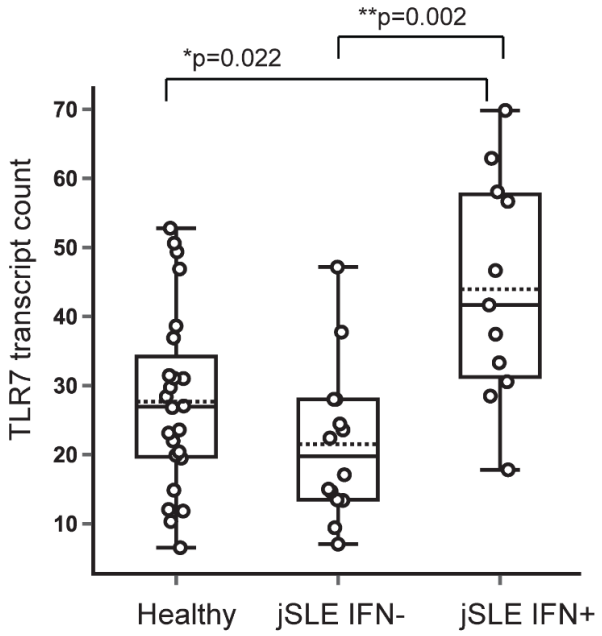


Figure 7-8. PBMC TLR7 gene expression was significantly higher in patients with IFN+ jSLE.
Volunteers with IFN+ jSLE had a higher PBMC *TLR7* gene expression than healthy volunteers (p=0.002) and those with IFN- jSLE (p=0.002) when analysed by linear regression, controlling for IFN score. (Healthy=24; jSLE IFN-=15, IFN+=14). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

7.4.2. *TLR9* and *RIG-1* gene expression were not increased in IFN+ or IFN- jSLE after controlling for IFN score.

It was shown in 6.4.2. that *TLR9* and *RIG-1* were both ISG. Therefore, when analysing for differences in expression of these genes, the effect of IFN score was controlled for through use of linear regression modelling. This showed that there was significantly less PBMC *TLR9* gene

expression in IFN- jSLE when compared to healthy volunteers (B=-6.602; p=0.027; 95% CI=-12.420, -0.784) after controlling for IFN score. There was no significant difference between healthy volunteers and IFN+ jSLE (B-=-2.236; p=0.604; 95% CI= -0.849, 6.378). *RIG-1* expression was lower in both IFN- (B=-34.879; p=0.111; 95% CI=-78.065, 8.307) and IFN+ (B=-64.669; p=0.048; 95% CI=-128.599, -0.738) jSLE when compared to healthy volunteers, but only reached statistical significance in IFN+ jSLE.

7.4.3. *LINE1* gene expression was higher in IFN+ jSLE after controlling for IFN score.

After correcting for IFN score and assessing by linear regression, it was seen that IFN+ jSLE had a significantly higher expression of *LINE1* than healthy volunteers (B=1564.395; p=0.007; 95% CI= 450.007, 2678.783) (Fig 7-9). There was no significant difference between healthy volunteers and IFN- jSLE (B= 473.585; p=0.212; 95% CI=-279.190, 1226.361).

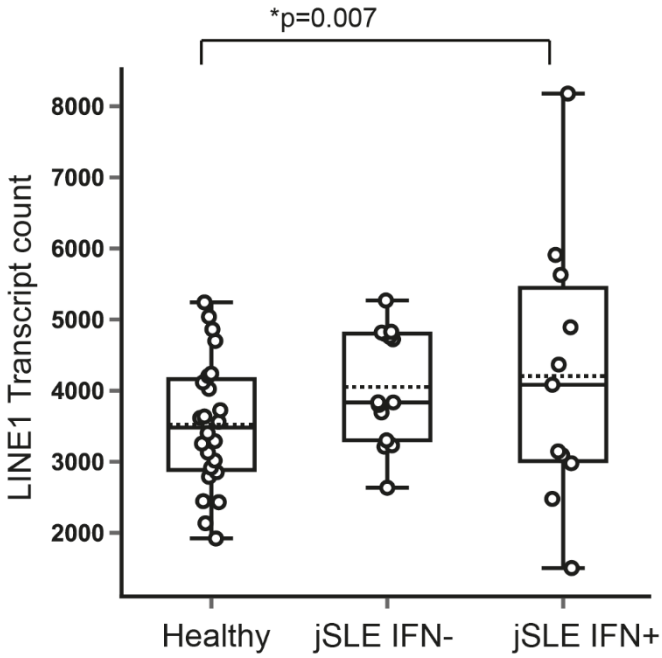


Figure 7-9. PBMC *LINE1* gene expression was significantly higher in patients with IFN+ jSLE.
Volunteers with IFN+ jSLE had a higher PBMC *LINE1* gene expression than healthy volunteers (p=0.007) when analysed by linear regression, controlling for IFN score. (Healthy=24; jSLE IFN-=15, IFN+=14). P values shown represent significance of the coefficient for the independent variable shown when analysed by linear regression

7.5. Discussion

There is an overabundance of ISG expression in jSLE which correlates with disease activity(189). More recently, ISG expression has been used to successfully stratify patients in trials of the anti- IFNα monoclonal antibody, sifalimumab (170) and the type 1 IFN receptor antagonist, anifrolumab(186) . These phase II trials have both shown a differential effect of therapy depending on whether the patient is 'IFN high' or 'IFN low'.

In this study, patients with low disease activity were specifically recruited. In this group, only half of the patients with jSLE were IFN+, by the relatively strict (and arbitrary) criteria of being >2SD from the mean of healthy individuals of roughly the same age. This is lower than previous studies in children which have reported that up to 80% of jSLE expresses an overall 'upregulated IFN signature'(189). This may be due to the deliberate selection of patients with low disease activity, but also due to the stringent criteria for an IFN+ classification in this study. Adult studies using this same criteria vary from a prevalence of 50% IFN+ patients in low disease activity studies, up to 75% 'IFN high' patients in moderate to severe disease activity studies(273).

It has been reported previously that jSLE is more prevalent, with a worse clinical phenotype and damage scores in non-white population groups (138, 140, 274, 275). In addition, there is a trend among adult studies that include stratification by IFN score for non-white SLE patients to be 'IFN-high' (273) and among gene expression data for there to be an increased IFN signature in non-white patients (200, 276, 277). The problem is that these studies, like the current data, are performed in Western cohorts and have proportional under-representation of non-white ethnic groups, despite the disease being least prevalent in white populations. Indeed, in these data, there was a non-significant trend for black patients to be 6 times more likely to be IFN+ as compared to white patients, which is interesting given that there were only 6 black patients recruited. If indeed there is a clinical difference in the therapeutic efficiency of drugs based on IFN score, then these ethnic differences will become important in therapeutic decision making and warrant further research. This finding needs to be confirmed in a larger cohort, which includes more non-white participants.

The only clinical variable that associated significantly with IFN score was the presence or absence of autoantibodies towards RNP in fitting with previous literature (201). Previous clinical associations have been seen between IFN score and disease activity measures such as SLEDAI(189). These were not seen in this study, probably due to the deliberate selection of low disease activity patients.

The IFN gene expression signature is difficult and time consuming to measure and standardise. In addition, serum IFNα cannot be measured in a clinical laboratory with any level of accuracy currently. If IFN score is to be used as a routine stratification measure in jSLE, there is a need for an easier, accurate measure of IFN activity. Tetherin is an anti-viral protein is expressed constitutively only on pDC and B cells but expressed widely upon IFN stimulation. In jSLE patients, among the tetherin expression in cells in the lymphocyte compartment, we found a strong correlation between CD4+ T cell tetherin expression and the IFN signature. CD4+ T cell

tetherin expression therefore may be a good target for future investigation as a clinical biomarker to distinguish between IFN high or IFN low jSLE, and more research is warranted in the future.

In addition to the clinical implications of stratifying jSLE patients by IFN score, we investigated whether stratification may lead to any further insights into type 1 IFN associated pathways in jSLE. To this end, it was seen that there were no significant differences in the percentage of pDC producing IFNα when stimulated with a TLR7 agonist, regardless of IFN stratification. There was still a significant decrease in the percentage of pDC producing IFNα upon TLR9 stimulation as compared to healthy controls, regardless of IFN score stratification. Once again, all these patients were on hydroxychloroquine, which may interfere with TLR7 and 9 functions. Regardless of that, stratification by IFN score did not change the trend seen in chapter 5, for a decreased pDC IFNα production in jSLE after TLR9 stimulation, but an unchanged production in jSLE after TLR7 stimulation.

There was however a difference in the PBMC gene expression of *TLR7* when stratified by IFN score. In chapter 6, it was reported that there was no significant difference in *TLR7* gene expression in jSLE as compared to healthy volunteers. Upon stratification however, a significantly higher *TLR7* gene expression was seen in IFN+ jSLE patients as compared to healthy volunteers and IFN- jSLE. It was confirmed in chapter 6 that *TLR7* was not IFN inducible, so this effect can be assumed to be not due to the general upregulation of ISG in jSLE. There is a well described pathogenic link between *TLR7* expression and SLE in mouse models (231, 232). These data raise the question of whether there is *TLR7* gene expression may be a pathological influence in SLE, depending on whether the disease is IFN+ or IFN-. *TLR7* may be a logical target for investigation in jSLE, but a stratified approach may be necessary.

Lastly, the significant over-expression of *LINE1* in IFN+ jSLE and not in IFN- jSLE, after correcting for IFN score was interesting. As stated in chapter 6, there is potential to target ERE RNA in jSLE to decrease the amount of RNA substrate available for *TLR7* stimulation. This also adds insight into the importance of stratifying this approach, based on the IFN score of the patient.

7.6. Conclusion

jSLE is a heterogeneous disease and stratifying by IFN gene expression score may be useful in assessing the pathophysiology of disease, as well as designing therapeutic targets. These data show that among baseline, relatively inactive disease, on relatively low steroid dose, roughly half of young people with jSLE have an IFN gene expression score that is significantly higher than healthy volunteers. Stratifying by IFN score has allowed us to show an over-expression of

TLR7 and *LINE1* RNA expression in IFN+ patients, which was not obvious when investigating the patients as a homogenous group.

Chapter 8: Conclusion and future work

This brief chapter will summarise the most important findings of this work and mention their importance, limitations and relevance to the field. New research questions arising from this work and future aims will be explored.

8.1. pDC were inherently more activated and expressed more anti-viral surface protein, tetherin in females as compared to males.

These data demonstrated for the first time in healthy young people that pDC were more activated in females (Fig. 4-3) and expressed more tetherin surface protein (Fig. 4-6). In this population, this related to the number of X chromosomes present, and not serum oestradiol or testosterone concentrations (Fig 4-4; 4-7). This is a remarkable observation, in *ex-vivo* cells from healthy young volunteers, where a basic demographic variable such as sex might not be expected to show a significant difference in the activation and priming of such a potent cell type. This underpins the importance of sex as a variable in biology and may explain some of the increased response of females to viral infection, vaccination and their predisposition towards jSLE. These findings now need to be confirmed in a separate population. In addition, pDC specific gene expression may be explored to assess whether these trends exist in the gene expression of pDC specifically.

8.2. Female pDC inherently produced more IFNα after TLR7 stimulation regardless of puberty.

These data show, for the first time in children and adolescents, that females had a higher percentage of pDC producing IFNα after TLR7 stimulation than males, regardless of pubertal status (Fig. 5-6). This effect was significant and substantial, especially given that these were healthy volunteers. It was demonstrated for the first time in humans, that the TLR7 induced production of type 1 IFN was significantly and substantially associated with the presence of two X chromosomes, regardless of serum oestradiol or testosterone concentrations (Fig. 5-9). The unique young human volunteers in this study allowed for a model that could uncouple the relationship between sex steroid and X chromosome number in humans. This implies that female pDC are not only inherently more activated than males, but also produce more type 1 IFN after TLR7 stimulation. This increased potential to react to RNA ligands adds to our understanding of why females have an enhanced antiviral and vaccine immune response. In jSLE, there is emerging opinion from the literature that an enhanced response to type 1 IFN may underpin pathogenesis. Therefore, if female pDC are inherently more capable of producing IFNα after RNA ligand stimulation, this may contribute to female's predisposition to develop jSLE.

8.3. Puberty and testosterone associated with more pDC producing IFNα after TLR7 stimulation

Remarkably, healthy volunteers who had completed puberty also showed a significant increase in the pDC production of IFNα specific to TLR7 stimulation when controlling for sex (Fig. 5-6). The human model revealed that serum testosterone associated positively with the TLR7 induced production of type 1 IFN in the presence of two X chromosomes, and negatively in the presence of one. Oestradiol has often been assumed to be responsible for sex differences in immunity, but in this population, controlling for oestradiol, testosterone showed the more significant effect. This is the first time that all of these variables have been analysed together in this manner and allow for the intriguing possibility that sex hormone may associate with immunity differently, depending on the number of X chromosomes present. These findings need to be confirmed in a separate, larger population group.

8.4. Females expressed more of the genes coding for RNA sensors than males

After observing that females had more activated pDC, with a higher tetherin expression, and produced more IFNα after TLR7 stimulation, it was further demonstrated that females expressed more of the genes coding for RNA-sensors *RIG-1* and *MDA5* (Fig. 6-4). This fits with the observation above that females seem more primed to detect RNA ligand than males. *TLR7* gene expression was increased in females, but only after puberty (Fig. 6-4). Surprisingly, *RIG-1* and *MDA5* gene expression associated with the number of X chromosomes whereas *TLR7*, which is coded for on the X chromosome, did not (Fig. 6-9). No explanation for this can be provided. Future research aims to focus on the pDC specific gene copy number of *TLR7* in females across puberty, to assess whether puberty associates with a dynamic increase in bi-allelic pDC expression of *TLR7*, and if true, explore underlying factors that may associate with this.

Conversely *TLR9* gene expression was lower in the post pubertal volunteers and associated with testosterone (Fig 6-5). *TLR7* gene expression has been shown to be important for lupus pathogenesis in murine models, whereas *TLR9* gene expression may be relatively protective. The observed increase in *TLR7* gene expression in females after puberty, along with a decrease in *TLR9* gene expression may contribute to the increased risk of females to develop jSLE around puberty.

8.5. TLR7 and TLR9 behave differently in jSLE

It was observed that TLR9 induced production of all measured cytokines, including IFNα, was decreased in jSLE as compared to healthy volunteers (Fig 5-13). Similarly, the relative gene expression of *TLR9* in jSLE was decreased after correcting for the IFN score (Fig. 7-8). Indeed, when jSLE was stratified by IFN score, there was a further significant decrease in the amount of IFNα produced after TLR9 stimulation in IFN+ patients (Fig. 7-7), suggesting negative feedback between TLR9 induced cytokine production and background IFNα. This is the first time that IFN gene expression score has been controlled for when assessing individual gene expression in this manner.

Conversely, the TLR7 induced production of IFNα was not different in low disease activity jSLE, although the TLR7 induced production of TNFα was increased (Fig. 5-12). In addition, the expression of *TLR7* gene was higher in males with jSLE compared to healthy males, but not altogether increased in low disease activity jSLE (Fig. 6-14). Interestingly, when stratified by IFN score, it was shown that IFN+ patients expressed significantly more *TLR7* gene than healthy volunteers or IFN- jSLE patients (Fig. 7-8). These experiments need to be extended to include patients with moderate to severe jSLE, to assess whether TLR7 induced IFNα production and *TLR7* gene expression remains the same or is increased as compared to healthy volunteers. It may stand to reason, that the appropriate response to a high background IFN would be a compensatory down-regulation in the expression and IFN producing capacity of TLR to prevent a sustained forward feedback loop, as is seen with TLR9. Therefore, the observation that *TLR7* gene expression is not changed overall, and increased in IFN+ jSLE is important. Future work is needed to assess whether *TLR7* gene expression and TLR7 induced IFNα production is appropriate in jSLE, given the high background IFN, and to explore whether TLR7 may be a valid target for future therapies.

8.6. Stratifying jSLE by IFN score

These data are the first to actively stratify young people with low disease activity based on background IFN induced gene expression score. It was observed that roughly half of the young people with jSLE were IFN+ based on this classification (Fig. 6-12), which was similar to adults with low to moderate disease activity SLE. It has previously been reported that jSLE shows an almost universal upregulated IFN gene signature, but this has not been observed here. In addition, stratifying by IFN score allowed for the observation that *TLR7* gene expression was increased in IFN+ disease, which was not the case for jSLE as a whole. Interestingly, the only ISG that was not relatively down-regulated in jSLE upon correcting for IFN score was the endogenous retroelement *LINE1* (Fig. 6-16; Fig. 7-9). This serves as an interesting pathway to investigate whether targeting the activity of *LINE1* may be beneficial in jSLE.

It was also shown that CD4 T cell tetherin surface protein expression correlated strongly with IFN score, and future work is necessary to assess whether this may serve as a useful biomarker for background type 1 IFN activity (Fig. 7-6).

Limitations of this study include the fact that these data were from a large population group and are broad and associative in nature. Further specific functional work, as mentioned above, is necessary to dissect these findings.

8.7. Conclusion

The original hypothesis to be tested was:

'Puberty associates with an increased TLR7 mediated type 1 IFN production in females.'

It has been shown that puberty and female sex associate with an increased TLR7 mediated type 1 IFN production in females and that this is related to X chromosome number and serum testosterone. Although the data suggests that *TLR7* gene expression and function is important in jSLE, a direct causal link has not been shown here. The role of TLR7 and TLR9 in jSLE, and the potential of TLR7 as a therapeutic target underpin future research in jSLE.

8.8 Future work.

- Establish whether pDC specific gene expression of ISG and other genes in the type 1 IFN pathway associate with sex and puberty, and change in jSLE, using the ongoing cohort of young people recruited to the Arthritis Research UK Centre for Adolescent Rheumatology. Briefly, pDC will be separated from PBMC by bead based magnetic cell separation and analysed for RNA gene expression using the same Nanostring assay.
- Investigate whether puberty associates with an increase in bi-allelic TLR7 gene expression in pDC in healthy females and assess for underlying chromosomal and hormonal factors that may associate with this. This would involve young people recruited to the above cohort and use allele-specific KASP (KBiosciences) PCR (as used in Souyris et al(127)) to discriminate the relative abundance of cells that are mono-allelic or bi-allelic for *TLR7.*
- Assess whether TLR7 induced IFNα production and *TLR7* gene expression is increased in severe to moderate disease activity jSLE as compared to healthy volunteers. This would include recruiting young people with higher disease activity jSLE from the above cohort and analysing by Nanostring.
- Assess whether background IFN stimulation associates with a change in TLR7 induced IFNα production and *TLR7* gene expression in healthy volunteers. This experiment

would include pre-stimulating PBMC with various dosages of IFNα for varying times, to assess whether this influences the subsequent production of IFNα or expression of *TLR7* gene.

- Investigate whether LINE1 copy number and retrotransposition is increased in jSLE by performing RNA sequencing on a larger cohort of young people with jSLE.
- Investigate, in a larger population of young people with jSLE, whether CD4 surface tetherin expression (as measured by flow cytometry) serves as a useful biomarker for high IFN signature disease.

References

1. Crow MK. Type I interferon in the pathogenesis of lupus. J Immunol. 2014;192(12):5459-68.
2. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nature Reviews Immunology. 2008;8(9):737.
3. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol. 2016;16(10):626-38.
4. Ambrose N, Morgan TA, Galloway J, Ionnoau Y, Beresford MW, Isenberg DA. Differences in disease phenotype and severity in SLE across age groups. Lupus. 2016;25(14):1542-50.
5. Murphy KKM, Murphy M. Janeway's immunobiology.2018.
6. Janeway CA, Jr. How the immune system works to protect the host from infection: a personal view. Proc Natl Acad Sci U S A. 2001;98(13):7461-8.
7. Gitlin D, Hitzig WH, Janeway CA. Multiple serum protein deficiencies in congenital and acquired agammaglobulinemia. J Clin Invest. 1956;35(11):1199-204.
8. Brubaker SW, Bonham KS, Zanoni I, Kagan JC. Innate immune pattern recognition: a cell biological perspective. Annu Rev Immunol. 2015;33:257-90.
9. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1-13.
10. Medzhitov R. Approaching the asymptote: 20 years later. Immunity. 2009;30(6):766-75.
11. Hashimoto C, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell. 1988;52(2):269-79.
12. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996;86(6):973-83.
13. Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem. 2000;275(7):4670-8.
14. Akira S, Takeda K. Toll-like receptor signalling. Nature Reviews Immunology. 2004;4(7):499.
15. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, et al. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. The Journal of Experimental Medicine. 2005;202(8):1131-9.
16. Berghöfer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 Ligands Induce Higher IFN-α Production in Females. The Journal of Immunology. 2006;177(4):2088-96.
17. Ghosh S, Klein RS. Sex drives dimorphic immune responses to viral infections. Journal of immunology (Baltimore, Md : 1950). 2017;198(5):1782-90.
18. Boasso A. Type I Interferon at the Interface of Antiviral Immunity and Immune Regulation: The Curious Case of HIV-1. Scientifica (Cairo). 2013;2013:580968.
19. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et al. The nature of the principal type 1 interferon-producing cells in human blood. Science. 1999;284(5421):1835-7.
20. McNally R, Eck MJ. JAK–cytokine receptor recognition, unboxed. Nature Structural & Molecular Biology. 2014;21(5):431.
21. Gibbert K, Schlaak JF, Yang D, Dittmer U. IFN-alpha subtypes: distinct biological activities in anti-viral therapy. Br J Pharmacol. 2013;168(5):1048-58.
22. Michalska A, Blaszczyk K, Wesoly J, Bluyssen HAR. A Positive Feedback Amplifier Circuit That Regulates Interferon (IFN)-Stimulated Gene Expression and Controls Type I and Type II IFN Responses. Front Immunol. 2018;9:1135.

23. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. Nature Reviews Immunology. 2015;15(2):87.
24. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, Van Royen-Kerkhoff A, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. New England Journal of Medicine. 2009;360(23):2426-37.
25. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. Annu Rev Immunol. 2014;32:513-45.
26. Rice GI, Melki I, Frémond ML, Briggs TA, Rodero MP, Kitabayashi N, et al. Assessment of Type I Interferon Signaling in Pediatric Inflammatory Disease. J Clin Immunol. 2017;37(2):123-32.
27. El-Sherbiny Y, Md Yusof MY, Hensor E, Wittmann M, Emery P, Vital E. FRI0308 Surface Tetherin Is A Novel Cell-Specific Biomarker for Interferon Response in Systemic Lupus Erythematosus. Annals of the Rheumatic Diseases. 2016;75(Suppl 2):546.
28. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. Nature Reviews Immunology. 2015;15(8):471.
29. Ori D, Murase M, Kawai T. Cytosolic nucleic acid sensors and innate immune regulation. Int Rev Immunol. 2017;36(2):74-88.
30. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science. 2013;339(6121):786-91.
31. Wu J, Sun L, Chen X, Du F, Shi H, Chen C, et al. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science. 2013;339(6121):826-30.
32. Zhao GN, Jiang DS, Li H. Interferon regulatory factors: at the crossroads of immunity, metabolism, and disease. Biochim Biophys Acta. 2015;1852(2):365-78.
33. Negishi H, Taniguchi T, Yanai H. The Interferon (IFN) Class of Cytokines and the IFN Regulatory Factor (IRF) Transcription Factor Family. Cold Spring Harb Perspect Biol. 2017.
34. Wu P, Wu J, Liu S, Han X, Lu J, Shi Y, et al. TLR9/TLR7-triggered downregulation of BDCA2 expression on human plasmacytoid dendritic cells from healthy individuals and lupus patients. Clinical Immunology. 2008;129(1):40-8.
35. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of Dendritic Cell Differentiation by IFN-α in Systemic Lupus Erythematosus. Science. 2001;294(5546):1540-3.
36. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20.
37. Facchetti F, Vermi W, Mason D, Colonna M. The plasmacytoid monocyte/interferon producing cells. Virchows Arch. 2003;443(6):703-17.
38. Abbott BP, Abbott R, Abbott TD, Abernathy MR, Acernese F, Ackley K, et al. GW150914: Implications for the Stochastic Gravitational-Wave Background from Binary Black Holes. Phys Rev Lett. 2016;116(13):131102.
39. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev. 2011;35(3):565-72.
40. Wald C, Wu C. Biomedical research. Of mice and women: the bias in animal models. Science. 2010;327(5973):1571-2.
41. Sawyer CC. Child mortality estimation: estimating sex differences in childhood mortality since the 1970s. PLoS Med. 2012;9(8):e1001287.
42. Flanagan KL, Jensen KJ. Sex Differences in Outcomes of Infections and Vaccinations in Under Five-Year-Old Children. In: Klein SL, Roberts CW, editors. Sex and Gender Differences in Infection and Treatments for Infectious Diseases. Cham: Springer International Publishing; 2015. p. 273-312.
43. Griesbeck M, Scully E, Altfeld M. Sex and gender differences in HIV-1 infection. Clin Sci (Lond). 2016;130(16):1435-51.
44. Schroder J, Kahlke V, Staubach KH, Zabel P, Stuber F. Gender differences in human sepsis. Arch Surg. 1998;133(11):1200-5.

45. Flanagan KL, Fink AL, Plebanski M, Klein SL. Sex and Gender Differences in the Outcomes of Vaccination over the Life Course. Annu Rev Cell Dev Biol. 2017;33:577-99.
46. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. Vaccine. 2008;26(29):3551-5.
47. Engler RJ, Nelson MR, Klote MM, VanRaden MJ, Huang CY, Cox NJ, et al. Half- vs full-dose trivalent inactivated influenza vaccine (2004-2005): age, dose, and sex effects on immune responses. Arch Intern Med. 2008;168(22):2405-14.
48. Cook MB, Dawsey SM, Freedman ND, Inskip PD, Wichner SM, Quraishi SM, et al. Sex disparities in cancer incidence by period and age. Cancer Epidemiol Biomarkers Prev. 2009;18(4):1174-82.
49. Cook MB, McGlynn KA, Devesa SS, Freedman ND, Anderson WF. Sex disparities in cancer mortality and survival. Cancer Epidemiol Biomarkers Prev. 2011;20(8):1629-37.
50. Lin PY, Sun L, Thibodeaux SR, Ludwig SM, Vadlamudi RK, Hurez VJ, et al. B7-H1-dependent sex-related differences in tumor immunity and immunotherapy responses. J Immunol. 2010;185(5):2747-53.
51. Dragin N, Bismuth J, Cizeron-Clairac G, Biferi MG, Berthault C, Serraf A, et al. Estrogen-mediated downregulation of AIRE influences sexual dimorphism in autoimmune diseases. 2016.
52. Zhu ML, Bakhru P, Conley B, Nelson JS, Free M, Martin A, et al. Sex bias in CNS autoimmune disease mediated by androgen control of autoimmune regulator. Nat Commun. 2016;7:11350.
53. Moore DJ. Sex (steroids) is in the AIRE. 2016.
54. Abdullah M, Chai P-S, Chong M-Y, Tohit ERM, Ramasamy R, Pei CP, et al. Gender effect on in vitro lymphocyte subset levels of healthy individuals. Cellular Immunology. 2012;272(2):214-9.
55. Lee BW, Yap HK, Chew FT, Quah TC, Prabhakaran K, Chan GS, et al. Age- and sex-related changes in lymphocyte subpopulations of healthy Asian subjects: from birth to adulthood. Cytometry. 1996;26(1):8-15.
56. Lisse IM, Aaby P, Whittle H, Jensen H, Engelmann M, Christensen LB. T-lymphocyte subsets in West African children: impact of age, sex, and season. J Pediatr. 1997;130(1):77-85.
57. Mori M, Adland E, Paioni P, Swordy A, Mori L, Laker L, et al. Sex Differences in Antiretroviral Therapy Initiation in Pediatric HIV Infection. PLoS One. 2015;10(7):e0131591.
58. Ruel TD, Zanoni BC, Ssewanyana I, Cao H, Havlir DV, Kamya M, et al. Sex differences in HIV RNA level and CD4 cell percentage during childhood. Clin Infect Dis. 2011;53(6):592-9.
59. Delmas MC, Jadand C, De Vincenzi I, Deveau C, Persoz A, Sobel A, et al. Gender difference in CD4+ cell counts persist after HIV-1 infection. SEROCO Study Group. AIDS. 1997;11(8):1071-3.
60. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. Genes Immun. 2009;10(5):509-16.
61. Afshan G, Afzal N, Qureshi S. CD4+CD25(hi) regulatory T cells in healthy males and females mediate gender difference in the prevalence of autoimmune diseases. Clin Lab. 2012;58(5-6):567-71.
62. Stoica G, Macarie E, Michiu V, Stoica RC. Biologic variation of human immunoglobulin concentration. I. Sex-age specific effects on serum levels of IgG, IgA, IgM and IgD. Med Interne. 1980;18(3):323-32.
63. Moxley G, Posthuma D, Carlson P, Estrada E, Han J, Benson LL, et al. Sexual dimorphism in innate immunity. Arthritis Rheum. 2002;46(1):250-8.
64. Aomatsu M, Kato T, Kasahara E, Kitagawa S. Gender difference in tumor necrosis factor-alpha production in human neutrophils stimulated by lipopolysaccharide and interferon-gamma. Biochem Biophys Res Commun. 2013;441(1):220-5.

65. Marriott I, Bost KL, Huet-Hudson YM. Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: a possible mechanism for gender-based differences in endotoxic shock susceptibility. J Reprod Immunol. 2006;71(1):12-27.
66. Ter Horst R, Jaeger M, Smeekens SP, Oosting M, Swertz MA, Li Y, et al. Host and Environmental Factors Influencing Individual Human Cytokine Responses. Cell. 2016;167(4):1111-24.e13.
67. Jaillon S, Berthenet K, Garlanda C. Sexual Dimorphism in Innate Immunity. Clin Rev Allergy Immunol. 2017.
68. Laffont S, Rouquié N, Azar P, Seillet C, Plumas J, Aspord C, et al. X-Chromosome Complement and Estrogen Receptor Signaling Independently Contribute to the Enhanced TLR7-Mediated IFN-α Production of Plasmacytoid Dendritic Cells from Women. The Journal of Immunology. 2014;193(11):5444-52.
69. Ziegler SM, Altfeld M. Human Immunodeficiency Virus 1 and Type I Interferons—Where Sex Makes a Difference. Frontiers in Immunology. 2017;8:1224.
70. LYON MF. Gene Action in the X-chromosome of the Mouse (Mus musculus L.). Nature. 1961;190(4773):372.
71. Jégu T, Aeby E, Lee JT. The X chromosome in space. Nature Reviews Genetics. 2017;18(6):377.
72. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nature Reviews Immunology. 2010;10(8):594.
73. Rocha STd, Heard E. Novel players in X inactivation: insights into. Nature Structural & Molecular Biology. 2017;24(3):197.
74. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature. 2005;434(7031):400-4.
75. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nature Reviews Immunology. 2010;10(8):594-604.
76. Bianchi I, Lleo A, Gershwin ME, Invernizzi P. The X chromosome and immune associated genes. J Autoimmun. 2012;38(2-3):J187-92.
77. Spolarics Z, Peña G, Qin Y, Donnelly RJ, Livingston DH. Inherent X-Linked Genetic Variability and Cellular Mosaicism Unique to Females Contribute to Sex-Related Differences in the Innate Immune Response. Front Immunol. 2017;8:1455.
78. Wang J, Syrett CM, Kramer MC, Basu A, Atchison ML, Anguera MC. Unusual maintenance of X chromosome inactivation predisposes female lymphocytes for increased expression from the inactive X. 2016.
79. Qu K, Zaba LC, Giresi PG, Li R, Longmire M, Kim YH, et al. Individuality and variation of personal regulomes in primary human T cells. Cell Syst. 2015;1(1):51-61.
80. Busque L, Paquette Y, Provost S, Roy DC, Levine RL, Mollica L, et al. Skewing of X-inactivation ratios in blood cells of aging women is confirmed by independent methodologies. Blood. 2009;113(15):3472-4.
81. Nielsen J, Wohlert M. Chromosome abnormalities found among 34,910 newborn children: results from a 13-year incidence study in Arhus, Denmark. Hum Genet. 1991;87(1):81-3.
82. Bondy CA. Care of girls and women with Turner syndrome: a guideline of the Turner Syndrome Study Group. J Clin Endocrinol Metab. 2007;92(1):10-25.
83. Cameron-Pimblett A, La Rosa C, King TFJ, Davies MC, Conway GS. The Turner syndrome life course project: Karyotype-phenotype analyses across the lifespan. Clin Endocrinol (Oxf). 2017;87(5):532-8.
84. Clinical manifestations and diagnosis of Turner syndrome - UpToDate: ing; 2018 [Available from: https://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-turner-syndrome.
85. White B, White BA, Porterfield SP. Endocrine and Reproductive Physiology E-Book: Mosby Physiology Monograph Series: Mosby; 2012.

86. Berne RM, Koeppen BM, Stanton BA, Levy MN. Berne & Levy physiology. 6th ed. Philadelphia: Mosby/Elsevier; 2008. xii, 834 p. p.
87. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. Immunol Lett. 2005;97(1):107-13.
88. Straub RH. The complex role of estrogens in inflammation. Endocr Rev. 2007;28(5):521-74.
89. Khan D, Ansar Ahmed S. The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. Front Immunol. 2015;6.
90. Fox HS, Bond BL, Parslow TG. Estrogen regulates the IFN-gamma promoter. J Immunol. 1991;146(12):4362-7.
91. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF, et al. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J Immunol. 2004;173(4):2227-30.
92. Tyagi AM, Srivastava K, Mansoori MN, Trivedi R, Chattopadhyay N, Singh D. Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis. PLoS One. 2012;7(9):e44552.
93. Pauklin S, Sernandez IV, Bachmann G, Ramiro AR, Petersen-Mahrt SK. Estrogen directly activates AID transcription and function. J Exp Med. 2009;206(1):99-111.
94. Jilma B, Department of Clinical Pharmacology UoV, Austria., Eichler HG, Department of Clinical Pharmacology UoV, Austria., Breiteneder H, Department of Clinical Pharmacology UoV, Austria., et al. Effects of 17 beta-estradiol on circulating adhesion molecules. The Journal of Clinical Endocrinology & Metabolism. 2018;79(6):1619-24.
95. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update. 2005;11(4):411-23.
96. Page ST, Plymate SR, Bremner WJ, Matsumoto AM, Hess DL, Lin DW, et al. Effect of medical castration on CD4+CD25+ T cells, CD8+ T cell IFN-γ expression, and NK cells: a physiological role for testosterone and/or its metabolites. https://doiorg/101152/ajpendo004842005. 2006.
97. @nhsuk. Early or delayed puberty - NHS: Department of Health; 2018 [Available from: https://www.nhs.uk/conditions/early-or-delayed-puberty/.
98. WHO | Adolescent health: World Health Organization; 2018 [updated 2018-10-23 02:33:15. Available from: https://www.who.int/topics/adolescent_health/en/.
99. Bordini B, Rosenfield RL. Normal Pubertal Development: Part I: The Endocrine Basis of Puberty. 2011.
100. Butler G, Kirk J. Paediatric Endocrinology and Diabetes (Oxford Specialist Handbooks in Paediatrics).
101. Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child. 1969;44(235):291-303.
102. Cole T, Butler G, Short J, Wright C, editors. Puberty phase specific growth charts: a radically new approach to the assessment of adolescent growth2012 2012-05-01: BMJ Publishing Group Ltd.
103. Growth charts | RCPCH 2018 [Available from: https://www.rcpch.ac.uk/resources/growth-charts.
104. Whincup PH, Gilg JA, Odoki K, Taylor SJC, Cook DG. Age of menarche in contemporary British teenagers: survey of girls born between 1982 and 1986. BMJ. 2001;322(7294):1095-6.
105. Perry JR, Day F, Elks CE, Sulem P, Thompson DJ, Ferreira T, et al. Parent-of-origin-specific allelic associations among 106 genomic loci for age at menarche. Nature. 2014;514(7520):92-7.
106. Kelly Y, Zilanawala A, Sacker A, Hiatt R, Viner R. Early puberty in 11-year-old girls: Millennium Cohort Study findings. 2016.

107. Normal puberty - UpToDate 2018 [Available from: https://www.uptodate.com/contents/normal-puberty.
108. Winter JS, Faiman C, Hobson WC, Prasad AV, Reyes FI. Pituitary-gonadal relations in infancy. I. Patterns of serum gonadotropin concentrations from birth to four years of age in man and chimpanzee. J Clin Endocrinol Metab. 1975;40(4):545-51.
109. Johannsen TH, Department of Growth and Reproduction R, University Hospital of Copenhagen, Copenhagen, Denmark, International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health R, University of Copenhagen, Copenhagen, Denmark, Main KM, Department of Growth and Reproduction R, University Hospital of Copenhagen, Copenhagen, Denmark, International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health R, University of Copenhagen, Copenhagen, Denmark, et al. Sex Differences in Reproductive Hormones During Mini-Puberty in Infants With Normal and Disordered Sex Development. The Journal of Clinical Endocrinology & Metabolism. 2018;103(8):3028-37.
110. Kuiri-Hanninen T, Sankilampi U, Dunkel L. Activation of the hypothalamic-pituitary-gonadal axis in infancy: minipuberty. Horm Res Paediatr. 2014;82(2):73-80.
111. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. 2015.
112. Barrett J. Gender dysphoria: assessment and management for non-specialists. Bmj. 2017;357:j2866.
113. Cartaya J, Lopez X. Gender dysphoria in youth: a review of recent literature. Curr Opin Endocrinol Diabetes Obes. 2018;25(1):44-8.
114. Diagnostic and Statistical Manual of Mental Disorders (DSM-5®)2018.
115. Butler G, De Graaf N, Wren B, Carmichael P. Assessment and support of children and adolescents with gender dysphoria. Arch Dis Child. 2018;103(7):631-6.
116. Costa R, Dunsford M, Skagerberg E, Holt V, Carmichael P, Colizzi M. Psychological Support, Puberty Suppression, and Psychosocial Functioning in Adolescents with Gender Dysphoria. J Sex Med. 2015;12(11):2206-14.
117. Santos-Ocampo AS. New onset systemic lupus erythematosus in a transgender man: possible role of feminizing sex hormones. J Clin Rheumatol. 2007;13(1):29-30.
118. Chan KL, Mok CC. Development of systemic lupus erythematosus in a male-to-female transsexual: the role of sex hormones revisited. Lupus. 2013;22(13):1399-402.
119. Wiik A, Andersson DP, Brismar TB, Chanpen S, Dhejne C, Ekström TJ, et al. Metabolic and functional changes in transgender individuals following cross-sex hormone treatment: Design and methods of the GEnder Dysphoria Treatment in Sweden (GETS) study. Contemp Clin Trials Commun. 102018. p. 148-53.
120. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences in the Toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1. Nat Med. 2009;15(8):955-9.
121. Seillet C, Laffont S, Trémollières F, Rouquié N, Ribot C, Arnal J-F, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor α signaling. Blood. 2012;119(2):454-64.
122. Ziegler SM, Beisel C, Sutter K, Griesbeck M, Hildebrandt H, Hagen SH, et al. Human pDCs display sex-specific differences in type I interferon subtypes and interferon alpha/beta receptor expression. Eur J Immunol. 2017;47(2):251-6.
123. Wang JP, Zhang L, Madera RF, Woda M, Libraty DH. Plasmacytoid dendritic cell interferon-α production to R-848 stimulation is decreased in male infants. BMC Immunology. 2012;13(1):35.
124. Wang JP, Zhang L, Madera RF, Woda M, Libraty DH. Plasmacytoid dendritic cell interferon-α production to R-848 stimulation is decreased in male infants. BMC Immunology. 2012;13:35-.

125. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A. 2013;110(9):3507-12.
126. Zhang Y, Castillo-Morales A, Jiang M, Zhu Y, Hu L, Urrutia AO, et al. Genes That Escape X-Inactivation in Humans Have High Intraspecific Variability in Expression, Are Associated with Mental Impairment but Are Not Slow Evolving. Mol Biol Evol. 2013;30(12):2588-601.
127. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, Grunenwald S, et al. TLR7 escapes X chromosome inactivation in immune cells. Science Immunology. 2018;3(19).
128. Tucker LB, Uribe AG, Fernandez M, Vila LM, McGwin G, Apte M, et al. Adolescent onset of lupus results in more aggressive disease and worse outcomes: results of a nested matched case-control study within LUMINA, a multiethnic US cohort (LUMINA LVII). Lupus. 2008;17(4):314-22.
129. Watson L, Leone V, Pilkington C, Tullus K, Rangaraj S, McDonagh JE, et al. Disease activity, severity, and damage in the UK Juvenile-Onset Systemic Lupus Erythematosus Cohort. Arthritis Rheum. 2012;64(7):2356-65.
130. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. The Journal of Experimental Medicine. 1978;147(6):1568-83.
131. Sthoeger ZM, Zinger H, Mozes E. Beneficial effects of the anti-oestrogen tamoxifen on systemic lupus erythematosus of (NZB×NZW)F1 female mice are associated with specific reduction of IgG3 autoantibodies. Annals of the Rheumatic Diseases. 2003;62(4):341-6.
132. Scofield RH, Bruner GR, Namjou B, Kimberly RP, Ramsey-Goldman R, Petri M, et al. Klinefelter's syndrome (47,XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome. Arthritis Rheum. 2008;58(8):2511-7.
133. Dillon S, Aggarwal R, Harding JW, Li LJ, Weissman MH, Li S, et al. Klinefelter's syndrome (47,XXY) among men with systemic lupus erythematosus. Acta Paediatr. 2011;100(6):819-23.
134. Stojan G, Petri M. Epidemiology of systemic lupus erythematosus: an update. Curr Opin Rheumatol. 2018;30(2):144-50.
135. Rees F, Doherty M, Grainge M, Davenport G, Lanyon P, Zhang W. The incidence and prevalence of systemic lupus erythematosus in the UK, 1999-2012. Ann Rheum Dis. 2016;75(1):136-41.
136. Tiffin N, Hodkinson B, Okpechi I. Lupus in Africa: can we dispel the myths and face the challenges? Lupus. 2014;23(1):102-11.
137. Rondina A, Watson AC. Bullous Sweet's syndrome and pseudolymphoma precipitated by IL-2 therapy. Cutis; cutaneous medicine for the practitioner. 2010;85(4):206-13.
138. Watson L, Leone V, Pilkington C, Tullus K, Rangaraj S, McDonagh JE, et al. Disease activity, severity, and damage in the UK juvenile-onset systemic lupus erythematosus cohort. Arthritis & Rheumatism. 2012;64(7):2356-65.
139. HIRAKI LT, BENSELER SM, TYRRELL PN, HARVEY E, HEBERT D, SILVERMAN ED. Ethnic Differences in Pediatric Systemic Lupus Erythematosus. 2009.
140. Lewandowski LB, Schanberg LE, Thielman N, Phuti A, Kalla AA, Okpechi I, et al. Severe disease presentation and poor outcomes among pediatric systemic lupus erythematosus patients in South Africa. Lupus. 2016.
141. Choi JH, Park DJ, Kang JH, Yim YR, Lee KE, Lee JW, et al. Comparison of clinical and serological differences among juvenile-, adult-, and late-onset systemic lupus erythematosus in Korean patients. Lupus. 2015;24(12):1342-9.
142. Torrente-Segarra V, Salman Monte TC, Rua-Figueroa I, Sanchez-Alonso F, Lopez-Longo FJ, Galindo-Izquierdo M, et al. Juvenile- and adult-onset systemic lupus erythematosus: a comparative study in a large cohort from the Spanish Society of Rheumatology Lupus Registry (RELESSER). Clin Exp Rheumatol. 2017;35(6):1047-55.
143. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40(9):1725.

144. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012;64(8):2677-86.
145. Sag E, Tartaglione A, Batu ED, Ravelli A, Khalil SM, Marks SD, et al. Performance of the new SLICC classification criteria in childhood systemic lupus erythematosus: a multicentre study. Clin Exp Rheumatol. 2014;32(3):440-4.
146. Classification criteria for SLE - UpToDate 2018 [Available from: https://www.uptodate.com/contents/image/print?imageKey=RHEUM%2F86633&topicKey=RHEUM%2F4668&source=see_link.
147. Tedeschi SK, Johnson SR, Boumpas D, Daikh D, Dörner T, Jayne D, et al. Developing and Refining New Candidate Criteria for SLE Classification: An International Collaboration. Arthritis Care Res (Hoboken). 2018;70(4):571-81.
148. Bell JM, Nair R, Solon A, Walker PD. SLE: the great imitator strikes again. Am J Kidney Dis. 2005;45(1):219-22.
149. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum. 1992;35(6):630-40.
150. Hay EM, Bacon PA, Gordon C, Isenberg DA, Maddison P, Snaith ML, et al. The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. Q J Med. 1993;86(7):447-58.
151. Liang MH, Socher SA, Larson MG, Schur PH. Reliability and validity of six systems for the clinical assessment of disease activity in systemic lupus erythematosus. Arthritis Rheum. 1989;32(9):1107-18.
152. Brunner HI, Feldman BM, Bombardier C, Silverman ED. Sensitivity of the Systemic Lupus Erythematosus Disease Activity Index, British Isles Lupus Assessment Group Index, and Systemic Lupus Activity Measure in the evaluation of clinical change in childhood-onset systemic lupus erythematosus. Arthritis Rheum. 1999;42(7):1354-60.
153. Gensous N, Marti A, Barnetche T, Blanco P, Lazaro E, Seneschal J, et al. Predictive biological markers of systemic lupus erythematosus flares: a systematic literature review. Arthritis Research & Therapy. 2017;19(1):238.
154. Furie RA, Petri MA, Wallace DJ, Ginzler EM, Merrill JT, Stohl W, et al. Novel evidence-based systemic lupus erythematosus responder index. Arthritis Rheum. 2009;61(9):1143-51.
155. Touma Z, Gladman DD, Ibanez D, Urowitz MB. Development and initial validation of the systemic lupus erythematosus disease activity index 2000 responder index 50. J Rheumatol. 2011;38(2):275-84.
156. Talk: Evaluation of Treatment Success in Systemic Lupus Erythematosus Clinical Trials: Development of the British Isles Lupus Assessment Group-Based Composite Lupus Assessment Endpoint (2011 ACR/ARHP Annual Scientific Meeting)2018.
157. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, Khamashta MA. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. Ann Rheum Dis. 2010;69(1):20-8.
158. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. J Immunol. 2011;186(8):4794-804.
159. Goldman FD, Gilman AL, Hollenback C, Kato RM, Premack BA, Rawlings DJ. Hydroxychloroquine inhibits calcium signals in T cells: a new mechanism to explain its immunomodulatory properties. Blood. 2000;95(11):3460-6.
160. Kaufmann AM, Krise JP. Lysosomal sequestration of amine-containing drugs: analysis and therapeutic implications. J Pharm Sci. 2007;96(4):729-46.
161. Sperber K, Quraishi H, Kalb TH, Panja A, Stecher V, Mayer L. Selective regulation of cytokine secretion by hydroxychloroquine: inhibition of interleukin 1 alpha (IL-1-alpha) and IL-6 in human monocytes and T cells. J Rheumatol. 1993;20(5):803-8.

162. Hu C, Lu L, Wan JP, Wen C. The Pharmacological Mechanisms and Therapeutic Activities of Hydroxychloroquine in Rheumatic and Related Diseases. Curr Med Chem. 2017;24(20):2241-9.
163. Barnes PJ. Glucocorticoids. Chem Immunol Allergy. 2014;100:311-6.
164. Baehr G, Soffer LJ. Treatment of Disseminated Lupus Erythematosus with Cortisone and Adrenocorticotropin*. Bull N Y Acad Med. 1950;26(4):229-34.
165. Kasturi S, Sammaritano LR. Corticosteroids in Lupus. Rheum Dis Clin North Am. 2016;42(1):47-62, viii.
166. Thakral A, Klein-Gitelman MS. An Update on Treatment and Management of Pediatric Systemic Lupus Erythematosus. Rheumatol Ther. 32016. p. 209-19.
167. Mina R, von Scheven E, Ardoin SP, Eberhard BA, Punaro M, Ilowite N, et al. Consensus treatment plans for induction therapy of newly diagnosed proliferative lupus nephritis in juvenile systemic lupus erythematosus. Arthritis Care Res (Hoboken). 2012;64(3):375-83.
168. Groot N, Graeff Nd, Avcin T, Bader-Meunier B, Brogan P, Dolezalova P, et al. European evidence-based recommendations for diagnosis and treatment of childhood-onset systemic lupus erythematosus: the SHARE initiative. 2017.
169. Mahieu MA, Strand V, Simon LS, Lipsky PE, Ramsey-Goldman R. A critical review of clinical trials in systemic lupus erythematosus. Lupus. 2016;25(10):1122-40.
170. Khamashta M, Merrill JT, Werth VP, Furie R, Kalunian K, Illei GG, et al. Sifalimumab, an anti-interferon-alpha monoclonal antibody, in moderate to severe systemic lupus erythematosus: a randomised, double-blind, placebo-controlled study. Ann Rheum Dis. 2016;75(11):1909-16.
171. Furie R, Khamashta M, Merrill JT, Werth VP, Kalunian K, Brohawn P, et al. Anifrolumab, an Anti-Interferon-α Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. Arthritis Rheumatol. 2017;69(2):376-86.
172. Update on TULIP 1 Phase III trial for anifrolumab in systemic lupus erythematosus: @astrazeneca; 2018 [Available from: https://www.astrazeneca.com/media-centre/press-releases/2018/update-on-tulip-1-phase-iii-trial-for-anifrolumab-in-systemic-lupus-erythematosus-31082018.html.
173. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum. 2010;62(1):222-33.
174. Merrill JT, Burgos-Vargas R, Westhovens R, Chalmers A, D'Cruz D, Wallace DJ, et al. The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 2010;62(10):3077-87.
175. Isenberg D, Gordon C, Licu D, Copt S, Rossi CP, Wofsy D. Efficacy and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). Ann Rheum Dis. 2015;74(11):2006-15.
176. Wallace DJ, Stohl W, Furie RA, Lisse JR, McKay JD, Merrill JT, et al. A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active systemic lupus erythematosus. Arthritis Rheum. 2009;61(9):1168-78.
177. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. Lancet. 2011;377(9767):721-31.
178. Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D, et al. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. Arthritis Rheum. 2011;63(12):3918-30.
179. Isenberg DA, Petri M, Kalunian K, Tanaka Y, Urowitz MB, Hoffman RW, et al. Efficacy and safety of subcutaneous tabalumab in patients with systemic lupus erythematosus: results

from ILLUMINATE-1, a 52-week, phase III, multicentre, randomised, double-blind, placebo-controlled study. Ann Rheum Dis. 2016;75(2):323-31.
180. Merrill JT, van Vollenhoven RF, Buyon JP, Furie RA, Stohl W, Morgan-Cox M, et al. Efficacy and safety of subcutaneous tabalumab, a monoclonal antibody to B-cell activating factor, in patients with systemic lupus erythematosus: results from ILLUMINATE-2, a 52-week, phase III, multicentre, randomised, double-blind, placebo-controlled study. Ann Rheum Dis. 2016;75(2):332-40.
181. Wallace DJ, Gordon C, Strand V, Hobbs K, Petri M, Kalunian K, et al. Efficacy and safety of epratuzumab in patients with moderate/severe flaring systemic lupus erythematosus: results from two randomized, double-blind, placebo-controlled, multicentre studies (ALLEVIATE) and follow-up. Rheumatology (Oxford). 2013;52(7):1313-22.
182. Wallace DJ, Kalunian K, Petri MA, Strand V, Houssiau FA, Pike M, et al. Efficacy and safety of epratuzumab in patients with moderate/severe active systemic lupus erythematosus: results from EMBLEM, a phase IIb, randomised, double-blind, placebo-controlled, multicentre study. Ann Rheum Dis. 732014. p. 183-90.
183. Clowse MEB, Wallace DJ, Furie RA, Petri MA, Pike MC, Leszczyński P, et al. Efficacy and Safety of Epratuzumab in Moderately to Severely Active Systemic Lupus Erythematosus: Results From Two Phase III Randomized, Double-Blind, Placebo-Controlled Trials. Arthritis Rheumatol. 692017. p. 362-75.
184. Wallace DJ, Strand V, Merrill JT, Popa S, Spindler AJ, Eimon A, et al. Efficacy and safety of an interleukin 6 monoclonal antibody for the treatment of systemic lupus erythematosus: a phase II dose-ranging randomised controlled trial. Ann Rheum Dis. 762017. p. 534-42.
185. Urowitz MB, Isenberg DA, Wallace DJ. Safety and efficacy of hCDR1 (Edratide) in patients with active systemic lupus erythematosus: results of phase II study. Lupus Sci Med. 2015;2(1):e000104.
186. Furie R, Khamashta M, Merrill JT, Werth VP, Kalunian K, Brohawn P, et al. Anifrolumab, an Anti–Interferon-α Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. Arthritis Rheumatol. 2017;69(2):376-86.
187. van Vollenhoven RF, Hahn BH, Tsokos GC, Wagner CL, Lipsky P, Touma Z, et al. Efficacy and safety of ustekinumab, an IL-12 and IL-23 inhibitor, in patients with active systemic lupus erythematosus: results of a multicentre, double-blind, phase 2, randomised, controlled study. Lancet. 2018;392(10155):1330-9.
188. Wallace DJ, Furie RA, Tanaka Y, Kalunian KC, Mosca M, Petri MA, et al. Baricitinib for systemic lupus erythematosus: a double-blind, randomised, placebo-controlled, phase 2 trial. Lancet. 2018;392(10143):222-31.
189. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and Granulopoiesis Signatures in Systemic Lupus Erythematosus Blood. The Journal of Experimental Medicine. 2003;197(6):711-23.
190. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, et al. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum. 1992;35(3):311-8.
191. Chen L, Morris DL, Vyse TJ. Genetic advances in systemic lupus erythematosus: an update. Curr Opin Rheumatol. 2017;29(5):423-33.
192. Tsokos GC, Lo MS, Reis PC, Sullivan KE. New insights into the immunopathogenesis of systemic lupus erythematosus. Nat Rev Rheumatol. 2016;12(12):716-30.
193. Kuo CF, Grainge MJ, Valdes AM, See LC, Luo SF, Yu KH, et al. Familial Aggregation of Systemic Lupus Erythematosus and Coaggregation of Autoimmune Diseases in Affected Families. JAMA Intern Med. 2015;175(9):1518-26.
194. Crow YJ, Manel N. Aicardi-Goutières syndrome and the type I interferonopathies. Nat Rev Immunol. 2015;15(7):429-40.
195. Hedrich CM. Epigenetics in SLE. Curr Rheumatol Rep. 2017;19(9).
196. Jeffries MA, Sawalha AH. Epigenetics in systemic lupus erythematosus: leading the way for specific therapeutic agents. Int J Clin Rheumtol. 2011;6(4):423-39.

197. Coit P, Jeffries M, Altorok N, Dozmorov MG, Koelsch KA, Wren JD, et al. Genome-wide DNA methylation study suggests epigenetic accessibility and transcriptional poising of interferon-regulated genes in naive CD4+ T cells from lupus patients. J Autoimmun. 2013;43:78-84.
198. Coit P, Yalavarthi S, Ognenovski M, Zhao W, Hasni S, Wren JD, et al. Epigenome profiling reveals significant DNA demethylation of interferon signature genes in lupus neutrophils. J Autoimmun. 2015;58:59-66.
199. Zhang Z, Song L, Maurer K, Petri MA, Sullivan KE. Global H4 acetylation analysis by ChIP-chip in systemic lupus erythematosus monocytes. Genes Immun. 2010;11(2):124-33.
200. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A. 2003;100(5):2610-5.
201. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum. 2005;52(5):1491-503.
202. Sharma S, Jin Z, Rosenzweig E, Rao S, Ko K, Niewold TB. Widely divergent transcriptional patterns between SLE patients of different ancestral backgrounds in sorted immune cell populations. Journal of Autoimmunity. 2015;60:51-8.
203. Weckerle CE, Franek BS, Kelly JA, Kumabe M, Mikolaitis RA, Green SL, et al. Network analysis of associations between serum interferon-alpha activity, autoantibodies, and clinical features in systemic lupus erythematosus. Arthritis Rheum. 2011;63(4):1044-53.
204. Ko K, Koldobskaya Y, Rosenzweig E, Niewold TB. Activation of the Interferon Pathway is Dependent Upon Autoantibodies in African-American SLE Patients, but Not in European-American SLE Patients. Front Immunol. 2013;4:309.
205. Niewold TB, Hua J, Lehman TJA, Harley JB, Crow MK. High serum IFN-[alpha] activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun. 2007;8(6):492-502.
206. Banchereau R, Hong S, Cantarel B, Baldwin N, Baisch J, Edens M, et al. Personalized Immunomonitoring Uncovers Molecular Networks that Stratify Lupus Patients. Cell. 2016;165(3):551-65.
207. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 2003;349(16):1526-33.
208. Kaur K, Zheng NY, Smith K, Huang M, Li L, Pauli NT, et al. High Affinity Antibodies against Influenza Characterize the Plasmablast Response in SLE Patients After Vaccination. PLoS One. 2015;10(5):e0125618.
209. Bonsignori M, Wiehe K, Grimm SK, Lynch R, Yang G, Kozink DM, et al. An autoreactive antibody from an SLE/HIV-1 individual broadly neutralizes HIV-1. J Clin Invest. 2014;124(4):1835-43.
210. Steinberg AD, Baron S, Talal N. THE PATHOGENESIS OF AUTOIMMUNITY IN NEW ZEALAND MICE, I. INDUCTION OF ANTINUCLEIC ACID ANTIBODIES BY POLYINOSINIC·POLYCYTIDYLIC ACID. 1969.
211. Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL, Notkins AL. Immune interferon in the circulation of patients with autoimmune disease. N Engl J Med. 1979;301(1):5-8.
212. Hua J, Kirou K, Lee C, Crow MK. Functional assay of type I interferon in systemic lupus erythematosus plasma and association with anti-RNA binding protein autoantibodies. Arthritis Rheum. 2006;54(6):1906-16.
213. Bell TM, Bansal AS, Shorthouse C, Sandford N, Powell EE. Low-titre auto-antibodies predict autoimmune disease during interferon-alpha treatment of chronic hepatitis C. J Gastroenterol Hepatol. 1999;14(5):419-22.
214. Ho V, Mclean A, Terry S. Severe systemic lupus erythematosus induced by antiviral treatment for hepatitis C. J Clin Rheumatol. 2008;14(3):166-8.

215. Wilson LE, Widman D, Dikman SH, Gorevic PD. Autoimmune disease complicating antiviral therapy for hepatitis C virus infection. Semin Arthritis Rheum. 2002;32(3):163-73.
216. Yao Y, Higgs BW, Morehouse C, de los Reyes M, Trigona W, Brohawn P, et al. Development of Potential Pharmacodynamic and Diagnostic Markers for Anti-IFN-α Monoclonal Antibody Trials in Systemic Lupus Erythematosus. Hum Genomics Proteomics. 2009;2009.
217. Shao WH, Cohen PL. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. Arthritis Res Ther. 132011. p. 202.
218. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860-921.
219. Mu X, Ahmad S, Hur S. Endogenous Retroelements and the Host Innate Immune Sensors. Adv Immunol. 2016;132:47-69.
220. Mavragani CP, Sagalovskiy I, Guo Q, Nezos A, Kapsogeorgou EK, Lu P, et al. Expression of Long Interspersed Nuclear Element 1 Retroelements and Induction of Type I Interferon in Patients With Systemic Autoimmune Disease. Arthritis Rheumatol. 2016;68(11):2686-96.
221. Attig J, Young GR, Stoye JP, Kassiotis G. Physiological and Pathological Transcriptional Activation of Endogenous Retroelements Assessed by RNA-Sequencing of B Lymphocytes. Front Microbiol. 2017;8:2489.
222. Smith CK, Kaplan MJ. The role of neutrophils in the pathogenesis of systemic lupus erythematosus. Curr Opin Rheumatol. 2015;27(5):448-53.
223. Kelly KM, Zhuang H, Nacionales DC, Scumpia PO, Lyons R, Akaogi J, et al. "Endogenous adjuvant" activity of the RNA components of lupus autoantigens Sm/RNP and Ro 60. Arthritis Rheum. 2006;54(5):1557-67.
224. Lövgren T, Eloranta M-L, Båve U, Alm GV, Rönnblom L. Induction of interferon-α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. Arthritis & Rheumatism. 2004;50(6):1861-72.
225. Tian J, Luo Y, Wu H, Long H, Zhao M, Lu Q. Risk of adverse events from different drugs for SLE: a systematic review and network meta-analysis. Lupus Sci Med. 2018;5(1):e000253.
226. Chamilos G, Gregorio J, Meller S, Lande R, Kontoyiannis DP, Modlin RL, et al. Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. Blood. 2012;120(18):3699-707.
227. Barrat FJ, Meeker T, Chan JH, Guiducci C, Coffman RL. Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms. Eur J Immunol. 2007;37(12):3582-6.
228. Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol. 2006;7(2):156-64.
229. Murphy ED, Roths JB. A Y chromosome associated factor in strain BXSB producing accelerated autoimmunity and lymphoproliferation. Arthritis Rheum. 1979;22(11):1188-94.
230. Andrews BS, Eisenberg RA, Theofilopoulos AN, Izui S, Wilson CB, McConahey PJ, et al. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. 1978.
231. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. Proc Natl Acad Sci U S A. 2006;103(26):9970-5.
232. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B Cell Responses to RNA-Related Antigens Due to TLR7 Gene Duplication. Science. 2006;312(5780):1669.
233. Fairhurst AM, Hwang SH, Wang A, Tian XH, Boudreaux C, Zhou XJ, et al. Yaa autoimmune phenotypes are conferred by overexpression of TLR7. Eur J Immunol. 2008;38(7):1971-8.

234. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity. 2006;25(3):417-28.
235. Santiago-Raber ML, Dunand-Sauthier I, Wu T, Li QZ, Uematsu S, Akira S, et al. Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice. J Autoimmun. 2010;34(4):339-48.
236. Celhar T, Yasuga H, Lee HY, Zharkova O, Tripathi S, Thornhill SI, et al. Toll-Like Receptor 9 Deficiency Breaks Tolerance to RNA-Associated Antigens and Up-Regulates Toll-Like Receptor 7 Protein in Sle1 Mice. Arthritis Rheumatol. 2018;70(10):1597-609.
237. Midgley A, Thorbinson C, Beresford MW. Expression of Toll-like receptors and their detection of nuclear self-antigen leading to immune activation in JSLE. Rheumatology (Oxford). 2012;51(5):824-32.
238. Komatsuda A, Wakui H, Iwamoto K, Ozawa M, Togashi M, Masai R, et al. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Exp Immunol. 2008;152(3):482-7.
239. Lyn-Cook BD, Xie C, Oates J, Treadwell E, Word B, Hammons G, et al. Increased expression of Toll-like receptors (TLRs) 7 and 9 and other cytokines in systemic lupus erythematosus (SLE) patients: ethnic differences and potential new targets for therapeutic drugs. Mol Immunol. 2014;61(1):38-43.
240. Kelley J, Johnson MR, Alarcon GS, Kimberly RP, Edberg JC. Variation in the relative copy number of the TLR7 gene in patients with systemic lupus erythematosus and healthy control subjects. Arthritis Rheum. 2007;56(10):3375-8.
241. García-Ortiz H, Velázquez-Cruz R, Espinosa-Rosales F, Jiménez-Morales S, Baca V, Orozco L. Association of TLR7 copy number variation with susceptibility to childhood-onset systemic lupus erythematosus in Mexican population. 2010.
242. Crowl JT, Gray EE, Pestal K, Volkman HE, Stetson DB. Intracellular Nucleic Acid Detection in Autoimmunity. Annu Rev Immunol. 2017;35:313-36.
243. Rice GI, del Toro Duany Y, Jenkinson EM, Forte GMA, Anderson BH, Ariaudo G, et al. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nature genetics. 2014.
244. Cederblad B, Blomberg S, Vallin H, Perers A, Alm GV, Rönnblom L. Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-alpha-producing cells. J Autoimmun. 1998;11(5):465-70.
245. Murayama G, Furusawa N, Chiba A, Yamaji K, Tamura N, Miyake S. Enhanced IFN-α production is associated with increased TLR7 retention in the lysosomes of palasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Res Ther. 2017;19(1):234.
246. Le Tortorec A, Willey S, Neil SJD. Antiviral Inhibition of Enveloped Virus Release by Tetherin/BST-2: Action and Counteraction. Viruses. 32011. p. 520-40.
247. Homann S, Smith D, Little S, Richman D, Guatelli J. Upregulation of BST-2/Tetherin by HIV Infection In Vivo. Journal of Virology. 2011;85(20):10659-68.
248. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis. 2007;66(5):589-98.
249. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol. 102009. p. 52.
250. Kim H, de Jesus AA, Brooks SR, Liu Y, Huang Y, VanTries R, et al. Development of a Validated Interferon Score Using NanoString Technology. J Interferon Cytokine Res. 2018;38(4):171-85.
251. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. NATURE BIOTECHNOLOGY.26(3):317-25.

252. Kulle A, Krone N, Holterhus PM, Schuler G, Greaves RF, Juul A, et al. Steroid hormone analysis in diagnosis and treatment of DSD: position paper of EU COST Action BM 1303 'DSDnet'. Eur J Endocrinol. 1762017. p. P1-9.
253. Moal V, Mathieu E, Reynier P, Malthiery Y, Gallois Y. Low serum testosterone assayed by liquid chromatography-tandem mass spectrometry. Comparison with five immunoassay techniques. Clin Chim Acta. 2007;386(1-2):12-9.
254. Rau FC, Dieter J, Luo Z, Priest SO, Baumgarth N. B7-1/2 (CD80/CD86) direct signaling to B cells enhances IgG secretion. J Immunol. 2009;183(12):7661-71.
255. Ohtomo T, Sugamata Y, Ozaki Y, Ono K, Yoshimura Y, Kawai S, et al. Molecular Cloning and Characterization of a Surface Antigen Preferentially Overexpressed on Multiple Myeloma Cells. Biochemical and Biophysical Research Communications. 1999;258(3):583-91.
256. Van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R, Johnson MC, et al. The Interferon-Induced Protein BST-2 Restricts HIV-1 Release and Is Downregulated from the Cell Surface by the Viral Vpu Protein. Cell Host & Microbe. 2008;3(4):245-52.
257. El-Sherbiny Y, Yusof MM, Hensor E, Psarras A, Mohamed A, Wittmann M, et al. A9.06 Analysis of cell-specific interferon response in systemic lupus erythematosus using a novel flow cytometric assay. Annals of the Rheumatic Diseases. 2016;75(Suppl 1):A72.
258. Jing Y, Shaheen E, Drake RR, Chen N, Gravenstein S, Deng Y. Aging is associated with a numerical and functional decline in plasmacytoid dendritic cells, whereas myeloid dendritic cells are relatively unaltered in human peripheral blood. Hum Immunol. 2009;70(10):777-84.
259. Griesbeck M, Ziegler S, Laffont S, Smith N, Chauveau L, Tomezsko P, et al. Sex Differences in Plasmacytoid Dendritic Cell Levels of IRF5 Drive Higher IFN-alpha Production in Women. J Immunol. 2015;195(11):5327-36.
260. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002;168(9):4531-7.
261. Cervantes JL, Weinerman B, Basole C, Salazar JC. TLR8: the forgotten relative revindicated. Cell Mol Immunol. 2012;9(6):434-8.
262. Zeuner RA, Klinman DM, Illei G, Yarboro C, Ishii KJ, Gursel M, et al. Response of peripheral blood mononuclear cells from lupus patients to stimulation by CpG oligodeoxynucleotides. Rheumatology (Oxford). 2003;42(4):563-9.
263. Kwok SK, Lee JY, Park SH, Cho ML, Min SY, Kim HY, et al. Dysfunctional interferon-alpha production by peripheral plasmacytoid dendritic cells upon Toll-like receptor-9 stimulation in patients with systemic lupus erythematosus. Arthritis Res Ther. 2008;10(2):R29.
264. Sacre K, Criswell LA, McCune JM. Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Res Ther. 2012;14(3):R155.
265. Rice GI, del Toro Duany Y, Jenkinson EM, Forte GM, Anderson BH, Ariaudo G, et al. Gain-of-function mutations in IFIH1 cause a spectrum of human disease phenotypes associated with upregulated type I interferon signaling. Nat Genet. 2014;46(5):503-9.
266. Jung JY, Suh CH. Incomplete clearance of apoptotic cells in systemic lupus erythematosus: pathogenic role and potential biomarker. Int J Rheum Dis. 2015;18(3):294-303.
267. Hung T, Pratt GA, Sundararaman B, Townsend MJ, Chaivorapol C, Bhangale T, et al. The Ro60 autoantigen binds endogenous retroelements and regulates inflammatory gene expression. Science. 2015;350(6259):455-9.
268. Klein SL, Hodgson A, Robinson DP. Mechanisms of sex disparities in influenza pathogenesis. J Leukoc Biol. 922012. p. 67-73.
269. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus - Komatsuda - 2008 - Clinical & Experimental Immunology - Wiley Online Library. 2018.
270. Shen N, Fu Q, Deng Y, Qian X, Zhao J, Kaufman KM, et al. Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. 2010.

271. Nickerson KM, Christensen SR, Shupe J, Kashgarian M, Kim D, Elkon K, et al. TLR9 regulates TLR7- and MyD88-dependent autoantibody production and disease in a murine model of lupus. J Immunol. 2010;184(4):1840-8.
272. Nickerson KM, Wang Y, Bastacky S, Shlomchik MJ. Toll-like receptor 9 suppresses lupus disease in Fas-sufficient MRL Mice. PLoS One. 2017;12(3):e0173471.
273. Kennedy WP, Maciuca R, Wolslegel K, Tew W, Abbas AR, Chaivorapol C, et al. Association of the interferon signature metric with serological disease manifestations but not global activity scores in multiple cohorts of patients with SLE. Lupus Sci Med. 2015;2(1).
274. González LA, Toloza SMA, McGwin G, Alarcón GS. Ethnicity in systemic lupus erythematosus (SLE): its influence on susceptibility and outcomes. Lupus. 2013;22(12):1214-24.
275. Borchers AT, Naguwa SM, Shoenfeld Y, Gershwin ME. The geoepidemiology of systemic lupus erythematosus. Autoimmun Rev. 2010;9(5):A277-87.
276. Iwamoto T, Dorschner J, Jolly M, Huang X, Niewold TB. Associations between type I interferon and antiphospholipid antibody status differ between ancestral backgrounds. Lupus Sci Med. 2018;5(1).
277. Ko K, Franek BS, Marion M, Kaufman KM, Langefeld CD, Harley JB, et al. Genetic Ancestry, Serum Interferon-α Activity, and Autoantibodies in Systemic Lupus Erythematosus. J Rheumatol. 2012;39(6):1238-40.